Metaplasticity Tunes Functional Plasticity in the Rodent Hippocampus

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von Qin Li
aus Henan / China

1. Referent:	Prof. Dr. Martin Korte
2. Referent:	Prof. Dr. Reinhard Köster
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‘SOMETIMES you will never know the true value of A MOMENT, until it becomes A MEMORY.’

Theodor Seuss Geisel

Dedicated To My Beloved Ones

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ABSTRACT

Synaptic plasticity, the activity-dependent modification of the strength of connections between neurons, is widely accepted to be the key component underlying the learning and memory machinery. Two major forms of persistent synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD) which refers to enduring strengthening or weakening of synaptic transmission efficiency respectively.

At the cellular basis, associative long-term memory are formed or maintained due to synergetic association of weakly and strongly activated synapses within a particular time frame, a process called “synaptic tagging and capture” (STC). STC provides a conceptual basis for how short-term memory is transformed to long-term memory in a time dependent manner. According to STC, a weak event marks the synapses with a local synaptic tag which captures the plasticity-related products (PRPs) from a nearby strong input, thus enabling the associativity between the two. Association of depressed and potentiated synapses can also occur in a synergetic manner, a positive associative interaction of LTP and LTD, which was coined “cross-tagging/cross-capture”. Under *in vitro* conditions, STC has been observed in the hippocampus for a limited time window of up to 1 h. Nevertheless, association of weak memory forms can occur far beyond this period and its mechanism is not well understood. In the present study I investigated the role of metaplasticity in tuning the synapses for coding long-term memory. Metaplasticity is the regulation of synaptic plasticity by prior neural activity of the same postsynaptic neuron or neural network that alters the threshold for plasticity. Here, metaplasticity induced by ryanodine receptor (RyR) activation or synaptic activation of metabotropic glutamate receptors (mGluRs) substantially prolongs the durability of the synaptic tag, thus extending the time window for associative interactions mediating storage of long-term memory. Intriguingly, RyR priming alters the synaptic tag setting process from a CaMKII-mediated (in non-primed STC) one to a protein kinase Mzeta (PKMζ)-mediated one (in primed STC). Furthermore, the PKMζ-mediated synaptic tag is resistant to depotentiation (DP), which indicates it is a durable, long-lasting and highly stable synaptic tag. Thus the association of weak synapses with strong synapses in the “late” stage of associative memory formation occurs only through processes of metaplasticity. My data also reveal that a short-lived, CaMKII-mediated tag may contribute to a mechanism for a fragile form of memory, while metaplasticity enables a PKMζ-mediated synaptic tag capable of prolonged interactions that induce a more stable form of memory that is resistant to stimuli that normally lead to a reversal of synaptic strength back to baseline values.

In Alzheimer’s disease (AD), synaptic plasticity impairment in the hippocampus is one of earliest events and the best neurobiological correlate of memory deficits in the progression of AD. In line with this, the present study indicates that not only the late phase of LTP (L-LTP) is impaired, but also STC and cross-capture are absent in the hippocampus of an AD transgenic mouse model of APP/PS1 mice *in vitro*. The current study investigated whether inducing metaplasticity through RyR activation in the neuronal networks of AD could prevent the degradation of synaptic memory. Here, priming RyR activation of the hippocampal synapses of APP/PS1 mice reverses the impaired L-LTP, leading to a long-lasting LTP that takes part in STC. In addition, RyR priming enables cross-capture in APP/PS1 mice. RyR priming exerts its effects through *de novo* protein synthesis of PKMζ. Notably, the primed L-LTP, STC as well as cross-capture in APP/PS1 mice resembles that of conventional ones, at least in the requirement for PKMζ activity for their maintenance. These findings indicate that a metaplastic upregulation of PKMζ might be able to compensate the synaptic plasticity deficits in AD, and by this means might be able to prevent or at least slow down memory loss.

ZUSAMMENFASSUNG

Die synaptische Plastizität, also die aktivitätsabhängige Modifikation der Stärke von Verbindungen zwischen Neuronen, ist weithin akzeptiert als die Schlüsselkomponente der Lern- und Gedächtnis-Maschinerie. Zwei Hauptformen der langanhaltenden, synaptischen Plastizität sind die Langzeit-Potenzierung (LTP) und die Langzeit-Depression (LTD), die sich jeweils auf die nachhaltige Verstärkung oder Schwächung der synaptischen Übertragungseffizienz beziehen.

Auf zellulärer Ebene werden assoziative Langzeit-Erinnerungen durch die synergetische Assoziation von Synapsen gebildet und erhalten, die innerhalb eines bestimmten Zeitrahmens schwach und stark aktiviert wurden. Dieser Prozess wird durch die „synaptic tagging and capture"-Hypothese (STC) beschrieben. STC liefert die konzeptionelle Basis für die zeitlich befristete Überführung von Kurzzeit-Erinnerungen in das Langzeit-Gedächtnis. Der STC zufolge markiert ein schwaches Ereignis die Synapsen mit einer lokalen synaptischen Markierung, die Plastizitäts-relevante Proteine (PRPs) von einem benachbarten, starken Input „einfängt", was die Assoziierung der beiden Ereignisse ermöglicht. Die Assoziierung von herabgesetzten und potenzierten Synapsen kann auch synergetisch erfolgen, durch eine positive assoziative Interaktion von LTP und LTD, die als „cross-tagging/cross-capture" bezeichnet wird. Unter in vitro Bedingungen konnten STC-Prozesse im Hippokampus für ein begrenztes Zeitfenster von bis zu einer Stunde beobachtet werden. Nichtsdestotrotz kann die Assoziierung von schwachen Erinnerungen lange über diese Periode hinaus erfolgen, aber dieser Mechanismus ist noch nicht hinreichend verstanden. In der vorliegenden Studie habe ich die Rolle der Metaplastizität bei der Abstimmung der Synapsen für die Kodierung von Langzeit-Erinnerungen untersucht. Metaplastizität bezeichnet die Regulierung der synaptischen Plastizität durch zuvor erfolgte neuronale Aktivität im gleichen postsynaptischen Neuron oder neuronalen Netzwerk, was zu einer Änderung des Grenzwertes für die Plastizität führt. In dieser Studie resultierte die Metaplastizität, erzeugt durch die Ryanodin-Rezeptor (RyR) Aktivierung oder durch die synaptische Aktivierung von metabotropen Glutamat-Rezeptoren (mGluRs), in einer deutlich verlängerten Beständigkeit der synaptischen Markierung. Dies führt zu einer Verlängerung des Zeitfensters für assoziative Wechselwirkungen, die zur Speicherung von Langzeit-Erinnerungen führen. Erstaun-licherweise ändert das RyR-Priming den synaptischen Markierungsprozess von einem CaMKII-vermittelten (nicht-vorbehandeltes STC) hin zu einem durch die Proteinkinase Mzeta (PKMζ) vermittelten Vorgang (vorbehandeltes STC). Des Weiteren ist die PKMζ-vermittelte synaptische Markierung resistent gegenüber Depotenzierungen (DP), was auf eine beständige, langanhaltende und sehr stabile synaptische Markierung hinweist. Somit erfolgt die Assoziierung von schwachen mit starken Synapsen in der späten Phase der assoziativen Gedächtnisbildung durch Prozesse der Meta-plastizität. Meine Daten zeigen weiterhin, dass eine kurzlebige, CaMKII-vermittelte Markierung an einem Mechanismus für eine fragile Form der Gedächtnisbildung beteiligt sein könnte. Im Gegensatz dazu ermöglicht die Metaplastizität eine PKMζ-vermittelte, zur verlängerten Interaktion fähige, synaptische Markierung. Diese induziert eine stabilere Form der Erinnerung, die resistent gegenüber jenen Stimulierungen ist, die zur Rückkehr der synaptischen Stärke zum Ausgangwert führen.

Bei der Alzheimer Erkrankung (AD) sind die Beeinträchtigungen der synaptischen Plastizität im Hippokampus eines der ersten Ereignisse und stellen das beste neurobiologische Korrelat für Gedächtnisdefizite im Zuge des Fortschreitens der Erkrankung dar. In Einklang damit weist die vor-liegende Studie darauf hin, dass nicht nur die späte Phase des LTP (L-LTP) beeinträchtig ist, sondern auch die STC und das „cross-capture" im Hippokampus im transgenen AD-Mausmodell der APP/ PS1-Mäuse *in vitro* nicht vorhanden sind. Des Weiteren behandelte meine Arbeit die Frage, ob die durch RyR-Aktivierung induzierte Metaplastizität in den neuronalen Netzwerken der AD den Abbau der synaptischen Erinnerung verhindern kann. Das RyR-Priming der hippokampalen Synapsen der APP/PS1-Mäusen führte zur Wiederherstellung des beeinträchtigen L-LTP, so dass das L-LTP im Rahmen der STC wieder ausgebildet wurde. Außerdem ermöglichte das RyR-Priming das „cross-capture" in APP/PS1-Mausen. Das RyR-Priming übt seinen Einfluss durch die *de novo* Proteinsyn-these von PKMζ aus. Insbesondere das vorbehandelte L-LTP, die STC sowie das „cross-capture" in APP/PS1-Mäusen gleichen denen konventioneller Tiere, soweit diese von einer PKMζ-Aktivierung für ihre Aufrechterhaltung abhängen. Diese Ergebnisse deuten darauf hin, dass eine metaplastische Hochregulierung der PKMζ die Defizite in der synaptischen Plastizität bei AD kompensieren könnte und auf diese Weise den Gedächtnisverlust verhindern oder zumindest verlangsamen könnte.

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LIST OF ABBREVIATIONS

Acronym	Definition
Aβ	amyloid β-peptide
ACSF	artificial cerebrospinal fluid
AD	Alzheimer's disease
AIDA	(R,S)-1-aminoindan-1,5,dicarboxylic acid
Arc	activity-regulated cytoskeleton-associated protein
AMPAR	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor
ANI	anisomycin
AP-5	D-2-amino-5-phosphonopentanoic acid
APP	amyloid precursor protein
BDNF	brain derived neurotrophic factor
CA	cornu ammonis
CaMKII	calcium/calmodulin-dependent protein kinase II
CICR	Ca^{2+}-induced Ca^{2+}-release
CREB	cAMP response element-binding
DG	dentate gyrus
DHPG	(R,S)-3, 5-dihydroxyphenylglycine
DP	depotentiation
EC	entorhinal cortex
E-LTP	early phase LTP
E-LTD	early phase LTD
ER	endoplasmic reticulum
ERK	extracellular-signal related kinase
fEPSP	field excitatory postsynaptic potential
GluA/R	glutamate receptor
HF	hippocampal formation
HFS	high frequency stimulation
KO	knockout
LFS	low-frequency stimulation
LTD	long-term depression
L-LTD	late phase LTD
LTP	long-term potentiation
L-LTP	late long-term potentiation

LTM	long-term memory
MAPK	mitogen-activated protein kinase
mGluR	metabotropic glutamate receptor
mTOR	mammalian target of rapamycin
NMDAR	N-methyl D-aspartate receptor
NSF	N-ethylmaleimide-sensitive factor
PICK1	protein interacting with C kinase-1
PIN1	protein interacting with NIMA1
PKA	protein kinase A
PKC	protein kinase C
PKMζ	protein kinase Mzeta
PLC	phospholipase C
PP1	protein phosphatase 1
PRPs	plasticity-related proteins/products
PS1	presenilin-1
RYA	ryanodine
RyR	ryanodine receptor
SLFS	strong low-frequency stimulation
SOCs	store-operated channels
STET	strong tetanization
STM	short-term memory
STP	short-term potentiation
TBS	theta-burst stimulation
WT	wild type
WTET	weak tetanization
ZIP	zeta inhibitory peptide

1 INTRODUCTION

1.1 Synaptic Plasticity

Humans as well as animals adapt to the ever-changing environment by life-long learning and memory processes. Learning, simply saying, is the behavior of acquiring new information or knowledge, whereas memory is the retention or expression of learned information. How those accumulating and large amounts of learned information leave their mark and being physiologically encoded and stored? This is one of the most significant challenges in neuroscience that we still seek to understand today. As early as in 1894, Santiago Ramon y Cajal proposed that information storage occurs in the central nervous system (CNS) as alterations in the strength of connections between neurons (i.e., synaptic plasticity) (Ramón y Cajal, 1894). This postulate is based on his anatomical studies that neurons communicate each other at specific "junctions" – later Charles S. Sherrington created the term "synapse" (Foster and Sherrington, 1897). The word "synapse" is derived from the Greek words "syn" and "haptein" that mean "together" and "to clasp" respectively (Tansey, 1997). The 10^{11} neurons in the brain are interconnected into neural circuitry by approximately 10^{14} synapses. Intriguingly, the structure and strength of synapses are not static but can be modified by specific pattern of neural activity. In late 1940s the Canadian psychologist Donald Hebb proposed that synaptic connections between cells can be strengthened provided simultaneous activation of cells occur – a coincidence-detector rule which is known as "*Hebb's Postulate*" (Hebb, 1949):

" When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased ".

"....any two cells or systems of cells that are repeatedly active at the same time will tend to become 'associated', so that activity in one facilitates activity in the other ".

- Hebb 1949

Hebb's postulate is a great advance that explains how neurons might adapt themselves to form engrams during learning and memory processes. Strikingly, this postulate was experimentally verified in 1973 by Timothy Bliss and Terje Lom who discovered that brief high electrical frequency stimulation of the perforant pathway-granule cell synapses to the rabbit hippocampus leads to a rapid and long-lasting increase of synaptic efficiency (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973), a phenomenon termed as long-term potentiation (LTP). However, Hebb's rule has limitations, as synapse follows this rule

would grow in strength without bound. In addition, there is no mechanism for connections to get weaker. The Bienenstock-Cooper-Munroe (BCM) model, designed to account for bidirectional regulation of synaptic strength, is an expansion of Hebb's theory (Bienenstock et al., 1982). According to this theory, synapses that are active when the postsynaptic cell is only weakly depolarized will undergo the opposite of LTP – long-term depression (LTD) (Bienenstock et al., 1982). LTD was demonstrated at the Schaffer collaterals-CA1 pyramidal cell synapses in the hippocampus by Lynch et al (Dunwiddie and Lynch, 1978). These bidirectional activity-dependent changes in synaptic strengthen such as LTP and LTD are referred to as synaptic plasticity. Accumulating evidences later on suggested that there is a potential link between synaptic plasticity and information processing which has been formalized by Morris and colleagues in 2000 as the "Synaptic Plasticity and Memory" (SPM) hypothesis (Martin et al., 2000):

Activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed.

Nowadays, it is widely accepted that synaptic plasticity such as LTP and LTD is an essential component of the cellular mechanisms that underlies the processes of learning and memory (Morris et al., 2003; Neves et al., 2008).

1.1.1 The Hippocampus

Hippocampus is the home team of synaptic plasticity. This mainly attributes to its unique structure and its intimate role in memory formation. The hippocampus is a prominent structure situated in the medial temporal lobes of the brain, flanked dorsally by the medial edge of the cortex and ventrally by the thalamus. Because of its elegant and curved structure which strongly resembles that of a seahorse, the name hippocampus is derived from the Greek term for seahorse (hippo = horse, kampos = sea monster). The hippocampus proper comprises three subdivisions of CA1, CA2 and CA3 (CA stand for cornu ammonis). The hippocampus proper, together with its several related cortical subregions, i.e., entorhinal cortex (EC), dentate gyrus (DG), subicular complex (subiculum, pre-and parasubiculum), form a functional system called hippocampal formation (HF).

Hippocampal Circuits

Firstly proposed by Ramón y Cajal in 1893, the hippocampus formation has a unique

intrinsic circuitry that is unidirectional and excitatory (Ramón y Cajal, 1894), different from that of most neocortical regions that are reciprocal (Felleman and Van Essen, 1991). The hippocampal interconnections are presented as a trisynaptic circuit or loop (Figure 1. 1). As an interface between hippocampus and cerebral cortex, the EC has a major source of inputs to the hippocampus, collecting highly processed sensory information from the visual, auditory, and somatic associative cortices through the way of the parahippocampal gyrus (PHG) and/or perirhinal cortex (PR). The axons of EC (superficial layer II) project to the DG and CA3 though the perforant pathway (PP). EC (layer III) neurons also project to CA1 and subiculum via the perforant and alvear pathways. Likewise, granule cells of DG project to CA3 via mossy fiber (MF) projections. Pyramidal neurons in the CA3 project to CA1 via Schaffer collaterals. Of note, the axons in this pathway come from CA3 neurons in both hemispheres, thus it is also named as Schaffer collateral-commissural pathway. Pyramidal cells in CA1 project to both the subiculum and the deep layers of the EC (layer V), providing the major output of the hippocampus. Intriguingly, the afferent fibers of hippocampal formation always run orthogonally to the apical dendritic axis to the pial surface, distinct from the other cortical regions where afferent fibers are radically oriented.

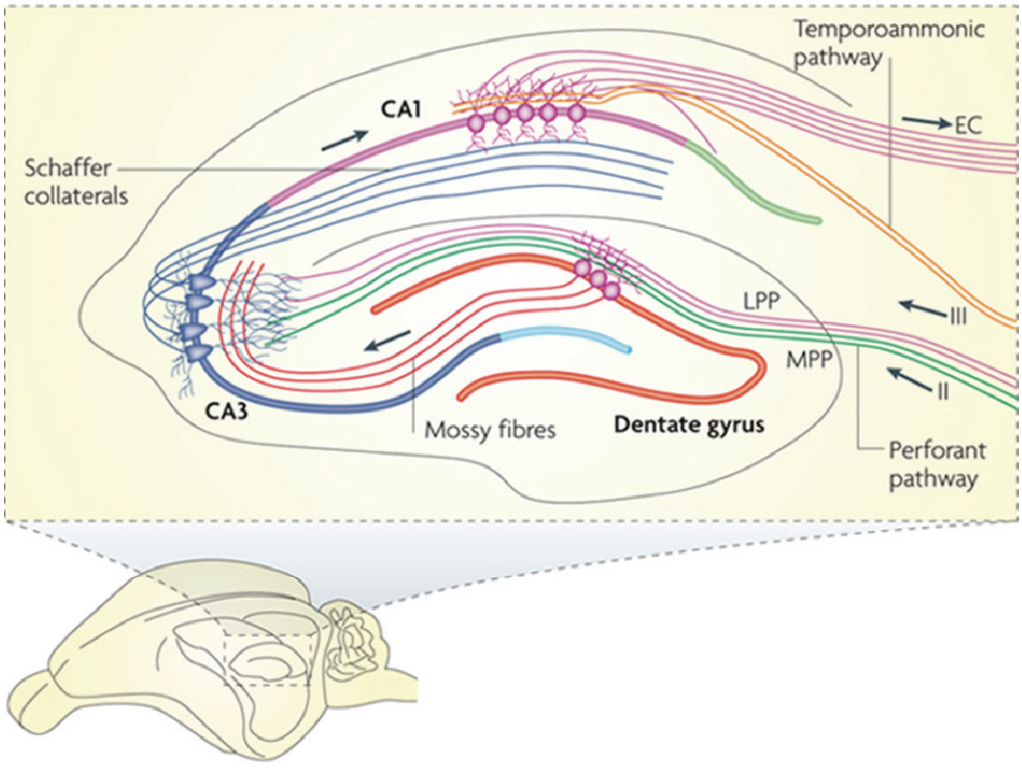


Figure 1. 1 | Schematic representation showing the hippocampal circuits.
Details see text 1.1.1. CA1, cornu ammonis 1; CA3, cornu ammonis 3; EC, entorhinal cortex; LPP, lateral perforant pathway; MPP, medial perforant pathway. (Source: Deng et al., 2010).

The main excitatory neurotransmitter along these pathways in the hippocampus is glutamate, as the same as that of in the other CNS. It activates three main classes of ionotropic glutamate receptor: NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid), and kainate receptors, named according to their selective agonists that activate them. NMDA receptors (NMDARs) and AMPA receptors (AMPARs) are ionotropic receptors that directly gate ion channels permeable to Na^+ and K^+, but they differ in that NMDARs are permeable to Ca^{2+} whereas AMPARs are not. During basal synaptic activity of excitatory synapses, synaptic released glutamate from the presynaptic terminal binds both NMDA receptor (NMDAR) and AMPA receptor (AMPAR), which are often colocalized on individual dendritic spines, leading to Na^+ influx through the AMPAR but not the NMDAR. Therefore, NMDAR contributes little to the basal synaptic transmission of excitatory synapses. This is due to the unique characteristic of NMDAR – Mg^{2+} blockade at resting membrane potential. However, Mg^{2+} block can be relieved by a depolarization of postsynaptic membrane by 20 mV or more. Once relieved, NMDAR allows the Na^+ and Ca^{2+} to enter into the postsynaptic cell which subsequently triggers the signal transduction pathways essential for synaptic plasticity that underlies hippocampal dependent learning and memory. Opening of NMDA receptor needs simultaneously the release of glutamate from the presynaptic neuron (presynaptic activation) and depolarization of the postsynaptic neuron (postsynaptic activation), thus it is referred as a coincidence detector (Bliss and Collingridge, 1993; Bourne and Nicoll, 1993).

Hippocampal Layers

In addition to its distinct circuits, the cells of the hippocampus are structured in clearly defined layers. All its principle cell populations (pyramidal cells in CA1 and CA3 subfields and granule cells in dentate gyrus) in the hippocampus condense tightly into single layers (Golgi et al., 2001). Furthermore, the CA regions are divided into four well defined layers. Pyramid-shaped soma of CA1 or CA3 neurons build the middle layer called the stratum pyramidale (s.p.). From the soma, two branching dendritic trees emerge. The basal dendrites form the stratum oriens (s.o.) building the outer layer. The apical dendrites are further divided into proximal dendrites and distal dendrites, with the proximal dendrites occupy the stratum radiatum (s.r.) forming the third inner layer and distal apical dendrites occupy the stratum lacunosum-moleculare (s.l.m.) that are defined as the forth inner layer.

Hippocampal Function

Over the years, a huge amount of studies have been undertaken in rodents and higher primates to seek the function of HF. Nowadays it is well accepted that the HF plays an important role in the formation of *declarative memory* (memory of *facts* and *events*). The idea comes from the direct evidence of a renowned human case study of patient H.M., who initially suffered from severely epileptic seizure. In an attempt to cure the epilepsy, he had a surgery at the age of 27 in which an 8 cm length of medial temporal lobe (including cortex, amygdala and anterior two-thirds of hippocampus) was bilaterally removed. Although the epileptic seizure was controlled, the surgery left him profound global amnesia. H.M. was incapable of remembering episodes experienced after the surgery (*anterograde amnesia*), coupled with partial *retrograde amnesia* (he was unable to recall information experienced back to 11 years preceding the operation), whereas his procedural learning ability and short-term memory (STM) was intact (Scoville and Milner, 1957). In 1971, O'Keefe and Dostrovsky discovered "place cells" in the hippocampus – a type of pyramidal neuron that fire when the animal enters a particular location in the environment (i.e., a single firing location), indicating that particular space can be encoded in the firing pattern of the hippocampus (O'Keefe and Dostrovsky, 1971). In 2005, May-Britt Moser and Edvard I. Moser discovered "grid cells" – nerve cells in medial entorhinal cortex (MEC) that fire at several spaced locations, forming a grid-like pattern (Hafting et al., 2005). The "place cells" and "grid cells" in the HF could encode the spatial information, allowing the mammals to remember the spatial location and the events experienced in the environment (Moser et al., 2008). In accords with those, studies on animal models using spatial memory tasks revealed that inactivation of the hippocampus by lesion, pharmacological inactivation or molecular knockout (KO) leads to either a failure to learn or deficits of *spatial memory* (i.e., part of declarative memory that is responsible for spatial locations) (Morris et al., 1986; Tsien et al., 1996; Martin et al., 2005; Pastalkova et al., 2006). Of note, the hippocampus has a time-limited role in the storage and retrieval of memory, i.e., memories are temporally retained in the hippocampus and then slowly transfer to the neocortex where they become permanently stored (*system consolidation*) (Squire and Alvarez, 1995; Dudai, 2004).

1.1.2 LTP and LTD in the Hippocampus

LTP and LTD, i.e., the long-term potentiation and depression of excitatory synaptic transmission, are wide-spread physiological phenomena expressed at almost all excitatory

synapses in the mammalian brain. Of note, the underlying mechanisms of LTP and LTD are not unitary but vary depending on the specific circuits in which they function. For instance, LTP at either perforant path-DG granule cell synapses or Schaffer collateral-CA1 pyramidal cell synapses is NMDAR-dependent, whereas that at mossy fiber-CA3 pyramidal cell synapses is NMDAR-independent. Among these forms, NMDAR-dependent LTP/LTD at Schaffer Collateral-CA1 synapses in the hippocampus is the most intensively studied and well-described form and appears to be identical to that observed at glutamatergic excitatory synapses throughout the mammalian brain.

Triggering Mechanisms

NMDAR-dependent LTP/LTD, by definition, requires synaptic activation of NMDARs by presynaptically released glutamate. In addition, it needs postsynaptic depolarization that leads to the relieve of Mg^{2+} blockade from the NMDAR channel, thereby allowing subsequently entry of both Ca^{2+} and Na^{+} into the postsynaptic spine (Figure 1. 2). The tight coincidence of pre and post synaptic activity indicates a Hebbian induction rule, and thus NMDAR-dependent synaptic plasticity is also named as Hebbian synaptic plasticity. The resultant elevation of Ca^{2+} in the postsynaptic dendrites activates the signal transduction cascades that are essential for LTP/LTD induction.

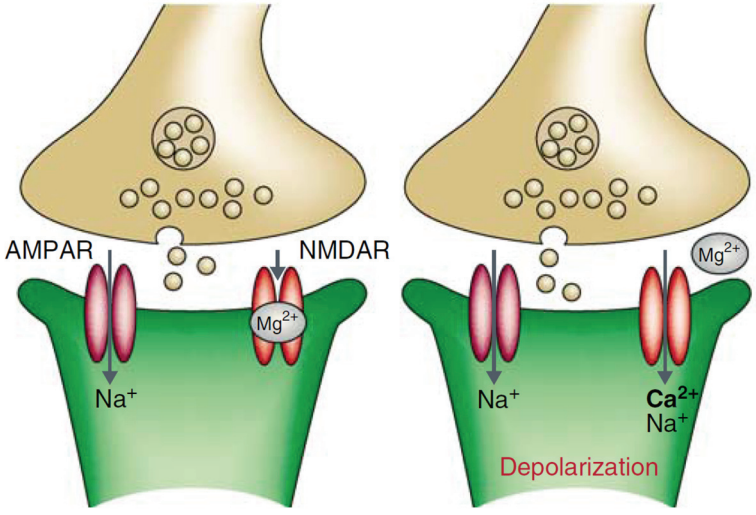


Figure 1. 2 | Model for the induction of LTP/LTD.
During normal synaptic transmission (left panel), synaptically released glutamate acts on both the NMDA receptors (NMDAR) and AMPAR receptors (AMPAR). However, Na^{+} flows only through the AMPAR channel but not the NMDAR channel because of the blockade of Mg^{2+}. Depolarization of the postsynaptic cell (right) relieves the Mg^{2+} block of the NMDAR channel, allowing both Na^{+} and Ca^{2+} to flow into the dendritic spine. The resultant elevation in Ca^{2+} within the dendritic spine is the critical trigger for LTP/LTD. (Source: Citri and Malenka, 2008).

But how can the same signal – Ca^{2+} entry through the NMDAR, triggers both LTP and

LTD? This difference attributes to the level of NMDAR activation or magnitude of the rise of [Ca^{2+}] (Mulkey and Malenka, 1992). LTP, experimentally, is induced by applying brief high-frequency stimulation (HFS) or low-frequency stimulation (LFS) in combination with strong postsynaptic depolarization (*pairing protocol*). HFS or the pairing protocol for LTP induction strongly depolarizes the postsynaptic membrane, which leads to a rapid and large amount of Ca^{2+} flood into postsynaptic dendrites ([Ca^{2+}] > 5 μM) (Malenka and Nicoll, 1993), high [Ca^{2+}] activate protein kinase such as calcium/calmodulin-dependent protein kinase II (CaMKII) (Fukunaga et al., 1993) and protein kinase C (PKC). CaMKII is an autophosphorylated protein kinase, i.e., once it is activated by calcium/calmodulin, it can be autophosphorylated at T286, by which its activity can be maintained after the dissociation of calcium/calmodulin (Lisman et al., 2002). Whereas the protocol for LTD induction typically involves "weaker" prolonged periods of low-frequency stimulation (LFS), which modestly depolarizes the postsynaptic neuron. As a result, there is only partial relief of Mg^{2+} blockade of NMDAR and thus a small amount of Ca^{2+} entry ([Ca^{2+}] ≤ 1 μM) (Cummings et al., 1996), low and prolonged [Ca^{2+}] activate protein phosphatases such as protein phosphatase 1 (PP1) – a key enzyme in LTD. Indeed, differential levels of buffering of intracellular Ca^{2+} have been shown to enable a transition between LTP and LTD (Nishiyama et al., 2000; Harney et al., 2006).

Expression Mechanisms

It is generally described that the expression of LTP and LTD involves tightly regulated trafficking of AMPARs into and out of synapses, respectively (Figure 1. 3) (Collingridge et al., 2004; Malenka and Bear, 2004; Citri and Malenka, 2008). The trafficking of AMPARs into and out of synapses is dynamically modulated by subunit-specific AMPAR interacting protein that have been implicated in the induction of NMDAR-dependent LTP/LTD (Anggono and Huganir, 2012). During the initiation of LTP, Ca^{2+} influx though NMDARs leads to the activation of various protein kinases such as CaMKII, PKC, protein kinase A (PKA) (Bliss and Collingridge, 1993). These protein kinases can catalyze the phosphorylation of GluR1 (glutamate receptor 1) at different sites. For example, CaMKII phosphorylates the AMPAR-binding protein stargazin, which causes stargazin to bind PSD95 (postsynaptic density protein 95), thereby increasing the number of AMPARs at the synapse (Hayashi et al., 2000; Lisman et al., 2012). In addition, PKC and PKA can phosphorylate Ser818 (Boehm et al., 2006) and Ser845 site (Oh et al., 2006), respectively, which promotes AMPARs insertion at the postsynaptic membrane. The expression of LTP

process also involves changes of biophysical properties of AMPARs – increase in the single-channel conductance driven by phosphorylation (Soderling and Derkach, 2000; Derkach et al., 2007). For instance, CaMKII phosphorylates AMPAR GluR1 subunits at S831, leading to a significant increase in single-channel conductance of homomeric GluR1 receptors (Derkach et al., 1999). The retention of AMPARs within the spine is mediated by the action of protein kinase Mzeta (PKMζ), a constitutively active atypical PKC isoform (Sacktor, 2011). PKMζ acts through the interaction of GluR2 (glutamate receptor 2) and the trafficking protein N-ethylmaleimide-sensitive factor (NSF; an ATPase that can stabilize GluR2-containing AMPARs at the synapse), and disruption of the GluR2-NSF interaction prevents LTP (Yao et al., 2008).

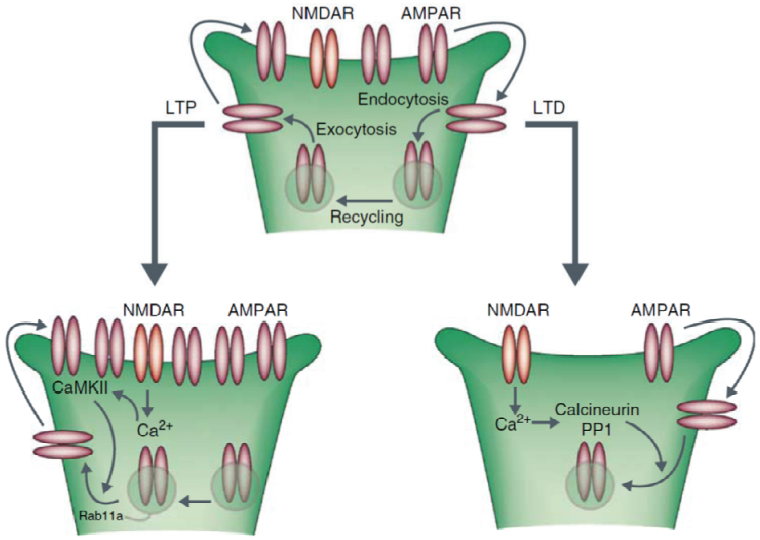


Figure 1. 3 | Model for the expression of LTP/LTD.
In the basal state (top), AMPARs in the postsynaptic membrane are continually being added and removed between the postsynaptic membrane and intracellular compartments through exocytosis and endocytosis, respectively. Following induction of LTP, activation of CaMKII due to Ca^{2+} influx though NMDARs enhances AMPARs exocytosis and stabilization at the synapse, leading to enhanced AMPARs insertion into the postsynaptic membrane. LTD expression involves internalization of postsynaptic AMPARs through calcineurin and PP1(protein phosphatase 1). (Source: Citri and Malenka, 2008).

The expression mechanism of LTD is the mirror image of that in LTP, i.e., activity-dependent dephosphorylation of AMPARs or endocytosis of AMPARs (Malenka and Bear, 2004; Massey and Bashir, 2007; Citri and Malenka, 2008; Collingridge et al., 2010). During the initiation of LTD, low and prolonged [Ca^{2+}] in the postsynaptic spine activate protein phosphatases such as PP1, which dephosphorylates its substrate ser845 on the AMPAR subunit GluR1. This dephosphorylation decreases AMPAR open channel probability and therefore reduces AMPAR-mediated transmission, contributing partially to

the expression of LTD. This is supported by the findings that mutant mice with knock-in alanines substitution of both Ser845 and Ser831 on GluR1 exhibit impaired NMDAR-LTD (Lee et al., 2003). Endocytosis of AMPARs also contributes to the expression of LTD. This is mediated by disruption of an interaction between GluR2 and NSF though the action of adaptor protein 2 (AP2) which has been suggested to displace NSF (Lee et al., 2002; Collingridge et al., 2004).

Maintenance Mechanisms

Long-lasting component of LTP (i.e., LTP that persist for hours, days, or even longer) requires new protein synthesis and gene transcription (Krug et al., 1984; Frey et al., 1988; Huang et al., 1994; Nguyen et al., 1994; Frey et al., 1996). The major mechanism underlies is that multiple intracellular signaling pathways activated during LTP induction such as PKA, calcium/calmodulin-dependent protein kinase IV (CaMKIV), and mitogen-activated protein kinase (MAPK) could phosphorylate and activate the key transcriptional factor cAMP response element-binding (CREB) protein (Abraham and Williams, 2003; Pittenger and Kandel, 2003; Malenka and Bear, 2004; Thomas and Huganir, 2004b) as well as immediate early genes (IEGs) such as c-Fos and zif268 (Davis et al., 2003). CREB mediates gene transcription though a Ca^{2+}/cAMP response element (CRE) on target genes, thereby stimulating the expression of transcriptional linked genes that are required for maintaining synaptic enhancement. Two possible CREB-responsive genes involved in LTP are Arc/Arg3.1 and PKMζ (Abraham and Williams, 2003; Hernandez et al., 2003). Arc (activity-regulated cytoskeleton-associated protein), a member of the IEG family, is expressed in the hippocampus and neocortex following LTP induction as well as learning tasks (Miyashita et al., 2008). It has been shown previously that Arc KO mice display impaired L-LTP and long-term memory (LTM) in several behavioral tasks (Plath et al., 2006). Notably, its mRNA is localized to activated synaptic sites in an NMDAR-dependent manner (Steward and Worley, 2001). PKMζ is synthesized over tens of minutes following LTP induction (Sacktor et al., 1993). The increased PKMζ in the strengthened spine could increase the number of functional AMPARs at PSD, thereby maintaining LTP from hours to even a day (Pastalkova et al., 2006; Sacktor, 2011).

> *"…it is possible that synthesis of specific proteins is the essential physical phenomenon paralleling memory, fantasy, and intuition. This hypothesis is supported by the fact that protein synthesis occurs in strongly stimulated neurons and that cells are able to 'learn' to synthesize new specific proteins…."*
>
> - Monné, 1948.

Consistently, disruption of PKMζ activity by its inhibitor such as ZIP (zeta inhibitory peptide) reverses established potentiation and long-term retention and storage of spatial information (Serrano et al., 2005; Pastalkova et al., 2006). In parallel, LTP maintenance is accompanied by structural remodeling including growth of new dendritic spines, enlargement of preexisting spines and their associated PSD (postsynaptic density) (Yuste and Bonhoeffer, 2001; Segal, 2005; Holtmaat and Svoboda, 2009).

LTD maintenance also depends on ongoing protein synthesis (Kauderer and Kandel, 2000; Manahan-Vaughan et al., 2000; Sajikumar and Frey, 2003), while transcription of new mRNA seems not necessary (Manahan-Vaughan et al., 2000). Morphologically, LTD maintenance involves shrinkage in the size of dendritic spines (Nagerl et al., 2004; Zhou et al., 2004; Holtmaat and Svoboda, 2009), which may due to the loss of AMPARs (Hsieh et al., 2006).

Properties of LTP/LTD

NMDAR-dependent LTP/LTD has several distinctive properties, including input-specificity, associativity and cooperativity (Reviewed by Bliss and Collingridge, 1993; Bear and Malenka, 1994; Malenka and Nicoll, 1999). I) *Input-specificity*. Once induced, LTP or LTD occurs specifically at the synapses that have received adequate stimulation rather than at all synapses on the same postsynaptic cell, even the neighboring synapses located only one micrometer or two away from the activated synapse remain unaffected. This feature is advantageous as it greatly increases the storage capacity of individual neurons in that different synapses on the same cell can be involved in separate circuits encoding different bits of information. II) *Associativity*. A weakly activated set of synapses that normally insufficient to produce LTP can become potentiated if it is paired with another set of adjacent strongly activated synapses on the same cell. The associativity property serves as a cellular analogue of associative or classical conditioning. Besides, it is an implicit property of the Hebbian synapses in that "*cells that fire together wire together*". III) *Cooperativity*. Cooperativity in nature is the spatial summation of EPSP of the activated synapse, which means that multiple afferent axons must be activated simultaneously to produce enough postsynaptic depolarization to cause LTP/LTD. Hence, a stimulus intensity threshold exists for inducing LTP/LTD. For instance, weak stimulus induce a posttetanic potentiation (PTP) with a duration of several seconds to minutes, intermediate weak stimulus gives rise to short-term potentiation (STP) lasting tens of several minutes, only strong activation induces long-lasting LTP that persists hours to days.

1.1.3 Distinct Temporal Phases of LTP and LTD

Similar with the memory consolidation process that has multiple stages: short-term memory (seconds to hours), long-term memory (hours to moths) as well as long-lasting memory (months to lifetime) (McGaugh, 2000), synaptic plasticity also displays distinct temporal phases or stages with different underlying mechanisms. In the case of LTP, it consists of several well defined phases based on the decay time constants (Figure 1. 4) (reviewed by Reymann and Frey, 2007; Citri and Malenka, 2008): I) *Posttetanic potentiation (PTP)* is the initial induction phase of LTP with duration of several seconds to minutes. II) *Short-term potentiation (STP)* is the short initial phase of LTP with a time course up to one hour. III) *Early-LTP (E-LTP)* is an early phase of LTP lasting less than a few hours. IV) *Late-LTP (L-LTP)*, a late component of LTP that lasts from several hours *in vitro* and weeks or months *in vivo*.

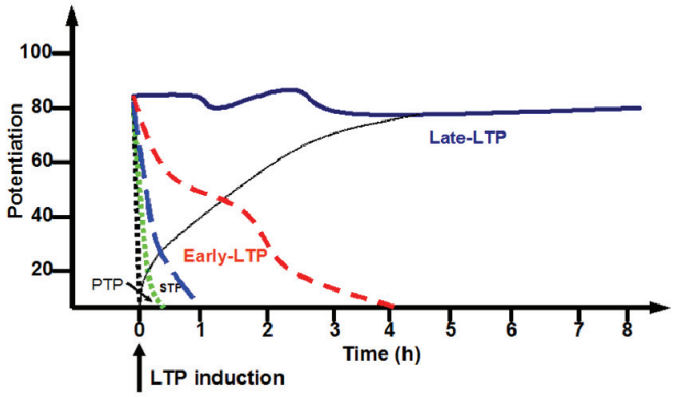


Figure 1. 4 | Schematic illustration of distinct temporal phases of LTP.
LTP consists of several well defined phases based on the decay time constants. Details see text 1.1.3. PTP, posttetanic potentiation; STP, short-term potentiation.

Mechanistically, the multiple phases of LTP are of significant differences. PTP is due to the transient enhancement of transmitter release in response to an action potential (AP) caused by increased Ca^{2+} concentration in the presynaptic nerve terminal during the stimulus trains, thus PTP is a presynaptic process (Zucker and Regehr, 2002). STP, induced by very weak tetanic stimulation with fewer stimulus numbers within one single train, is dependent on NMDAR activation that activated by local protein kinases such as CaMKII and tyrosine kinase but requires no protein synthesis (Dobrunz et al., 1997; Huang, 1998b). E-LTP and L-LTP share common property in that both of them need the activation of NMDAR and Ca^{2+} as the second messenger to trigger signal cascades for expression. However, they

differ from each other in the following aspects: I) *Induction*: a single tetanic stimulation of an afferent pathway lead to the E-LTP while repeated (three or more) tetanic trains stimulation delivered at time spaced of 5-10 min produce the more persistent L-LTP (Huang and Kandel, 1994; Kelleher et al., 2004). Of note, a single tetanus could also lead to L-LTP if the stimulation intensity and number of stimuli per tetanus are beyond some critical value (Bortolotto and Collingridge, 2000; Sajikumar et al., 2008). II) *Requirement of protein synthesis and transcription* (see Figure 1. 5): the maintenance of E-LTP depends on post-translational modification or trafficking of existing proteins and is insensitive to both protein synthesis and transcription inhibitors, whereas the persistence of L-LTP requires both transcription and protein synthesis (Frey et al., 1993; Nguyen et al., 1994; Huang, 1998a). It is worthy to notice that E-LTP is a parallel and independent phase of LTP expression that is not required for L-LTP expression (McGaugh, 2000; Reymann and Frey, 2007). All these multiple phases of LTP have been demonstrated in both the freely moving animals and hippocampal slices *in vitro*.

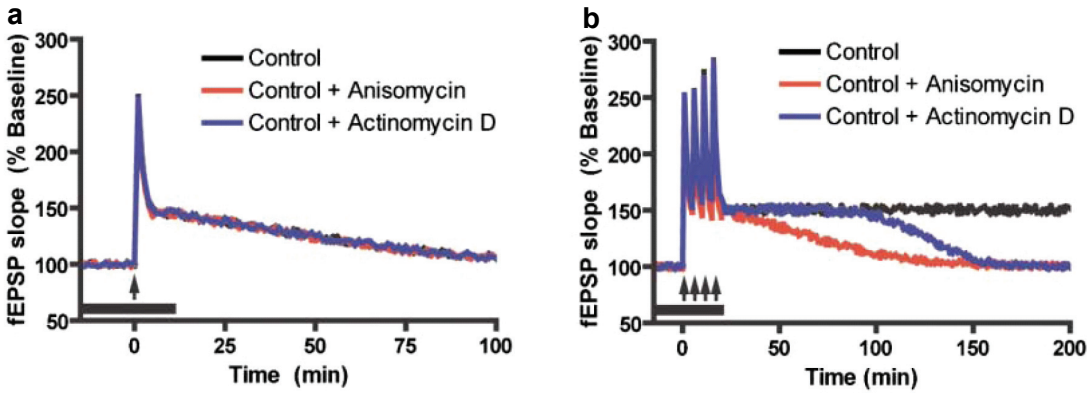


Figure 1. 5 | Properties of E-LTP and L-LTP.
(a) E-LTP (black line), lasting for about 1 h, is induced by a single train of tetanic stimulation (arrow). E-LTP is insensitive to translational inhibition (anisomycin) and transcriptional inhibition (actinomycin-D), indicating it is both translation and transcription independent. The solid bar represents the time period of inhibitor treatment. **(b)** The long-lasting L-LTP (black line) is triggered by four spaced trains of tetanic stimulation (arrows). Application of anisomycin prevents the persistence of L-LTP, leading to a decremental potentiation that resembles E-LTP. Actinomycin-D has no effect on the initial 60-90 min of LTP, but decreases the potentiation gradually afterwards. The solid bar represents the time of inhibitor treatment. (Source: Kelleher et al., 2004).

An extension of this categorization was proposed by Abraham and Otani in 1991, based on the difference in the decay time constants and molecular mechanisms. Here, LTP is classified into LTP1, LTP2, and LTP3. LTP1 is equivalent to E-LTP. LTP2 is an intermediate phase of L-LTP that depends on protein synthesis but is transcription independent, whereas LTP3 is equivalent to L-LTP that requires both transcription and

protein synthesis (Abraham and Otani, 1991; Raymond, 2007).

The similar property can be applied with LTD, with a transient protein synthesis independent early phase of LTD (E-LTD) and more persistent and protein synthesis dependent late phase of LTD (L-LTD). E-LTD can be induced by weak low frequency stimulation (WLFS) consisting of 900 pulses and lasts less than 2-3 h, whereas L-LTD is long-lasting as that of L-LTP and can be elicited by strong low frequency stimulation (SLFS) consisting of 2700 pulses (Sajikumar and Frey, 2003). Similar with LTP, E-LTD is protein synthesis-independent while L-LTD depends on ongoing protein synthesis (Kauderer and Kandel, 2000; Manahan-Vaughan et al., 2000; Sajikumar and Frey, 2003).

1.2 The Synaptic Tagging and Capture Hypothesis

During the late phase of LTP, the potentiated synapses need to be maintained by the delivery of newly synthesized proteins and mRNA transcription that mainly occurs at the cell body. But how those new mRNA and proteins used to stabilize the synaptic strength are selectively targeted to the cell's dendritic branches that have received the appreciate stimuli without known elaborate intracellular trafficking? There are several proposals seeking to explain the question, for instance, "mail hypothesis," "local protein synthesis hypothesis", "sensitization hypothesis" and "synaptic tagging and capture (STC) hypothesis"(reviewed by Frey and Morris, 1998b; Kelleher et al., 2004).

I) The *"mail hypothesis"* proposes that the newly synthesized mRNAs encoding the essential plasticity proteins, at the time of their born, are given a "synaptic address" to which they are transported. However, it has limitations as it cannot explain the early phase or transcription-independent LTP/LTD, which needs the preexisting mRNAs targeting to the activated synapses.

II) The *"local protein synthesis hypothesis"* postulates that activated synapses synthesize proteins locally (in the dendrite) and use locally, and mRNA targeting to the activated synapse are not needed (Schuman, 1997). Although the hypothesis is supported by findings that polyribosomes exist in single spines (Torre and Steward, 1996), it is biochemically expensive and cannot explain the heterosynaptic associations of LTP observed by Frey in 1997 (Frey and Morris, 1997).

III) The *"sensitization hypothesis"* suggests that plasticity-related macromolecules are

distributed to every synapse of the cell but at variable levels and their general availability at a given location influence the degree of the plasticity change. Nevertheless, this would have the effects of altering the threshold at which synaptic activation gives rise to lasting synaptic changes (Malinow et al., 2000).

IV) The *"synapse tagging and capture hypothesis"* asserts that the input specificity of L-LTP is achieved through two dissociable synaptic events (see Figure 1. 7). The first event involves the setting of synaptic tag at the potentiated synapses, which is transient and protein-synthesis independent. The second event comprises the synthesis of plasticity related products (PRPs) in the soma or local dendritic domains as a result of L-LTP induction. The tagged synapses, but not neighboring synapses, would be able to "capture" PRPs, leading to L-LTP persistence. In this manner, no elaborate targeting of synapse specific protein/mRNA is needed. Up to date, this hypothesis has been independently verified in various laboratories.

1.2.1 Synaptic Tagging and Capture

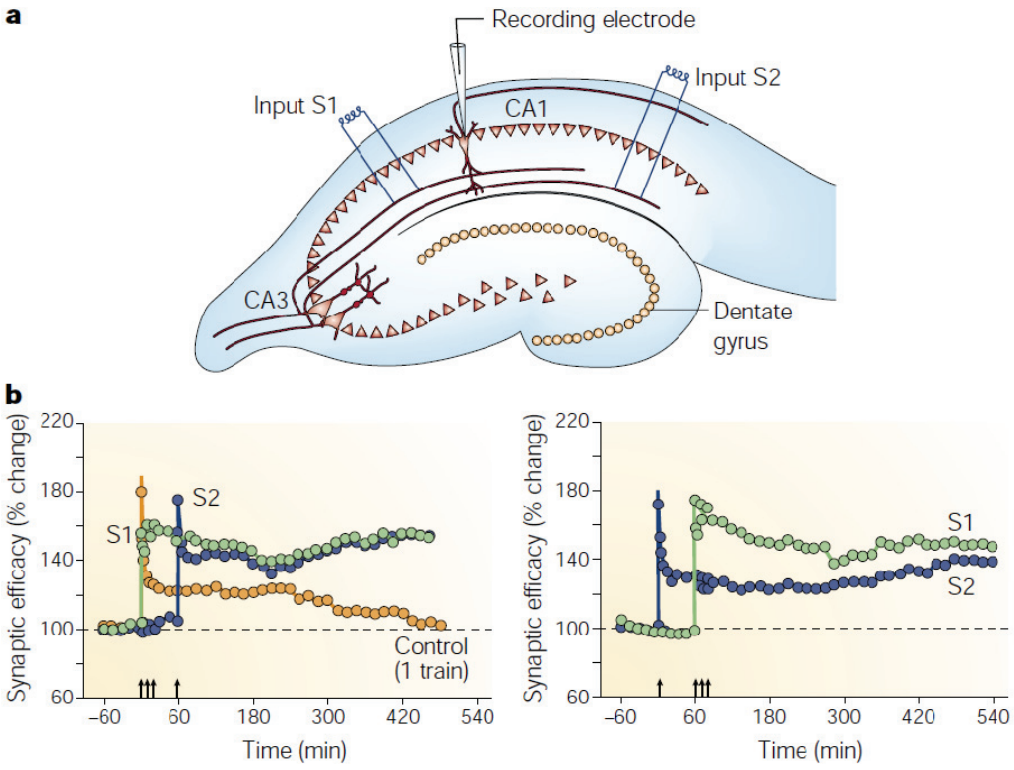


Figure 1. 6 | Synaptic tagging and capture in rodent hippocampal neurons.
(a) Two-pathway recordings in a hippocampal slice. **(b)** A single train of high-frequency stimulation (HFS) to one synaptic input produces an E-LTP that decays after 1.5 h (control, yellow circles). Three trains of HFS produce an L-LTP that persists for at least 8 h (green circles). If a single train is given to S2 either before (right panel) or after (left panel) three tetanic stimuli are applied to S1, persistent LTP occurs in both pathways. (Source: Martin and Kosik, 2002).

The STC hypothesis was based on the experimental evidences of two-pathway hippocampal slices recordings (i.e., a kind of experimental paradigm that stimulating two independent synaptic inputs but converge to the same population of neurons in the hippocampus) in Schaeffer collateral pathway *in vitro* (details see Figure 1. 6) (Frey and Morris, 1997). Briefly, a single train of high-frequency stimulation (HFS) that induces protein synthesis independent E-LTP at synaptic input 2 (S2) can be consolidated into L-LTP, provided repeated HFS has been or will be applied at synaptic input 1 (S1) within a limited time window. These observations can be explained by the STC hypothesis (see also Figure 1. 7): S2 by itself could not lead to protein synthesis as it received tetanic stimulation that induce E-LTP but could form a "tag" which can "capture" the newly synthesized proteins induced by the S1, establishing L-LTP. Whereas L-LTP induced in S1 supplied the necessary proteins not only for itself but also for S2 (E-LTP).

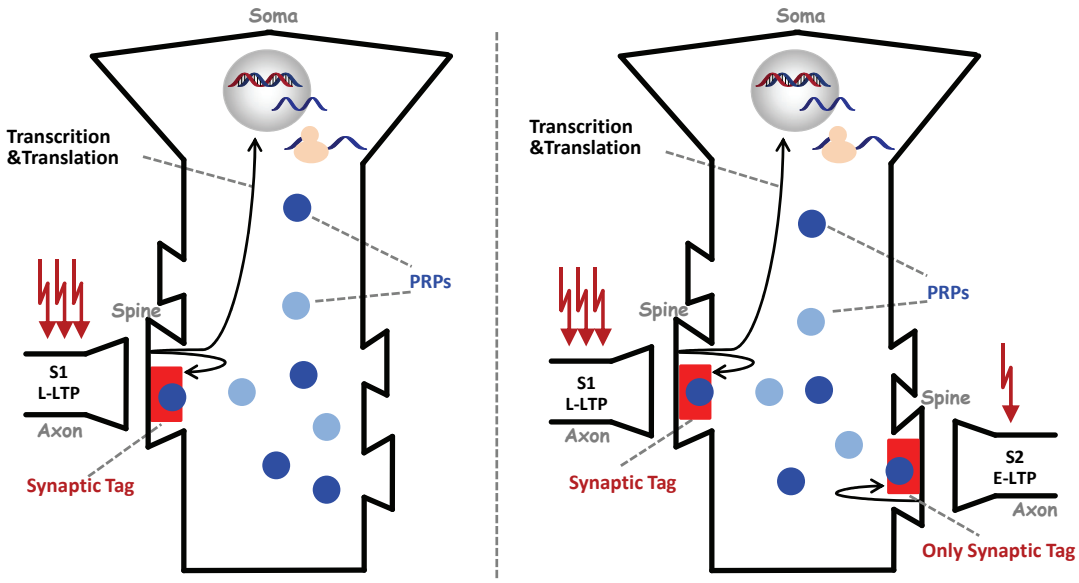


Figure 1. 7 | Model of synaptic tagging and capture (STC).
Left panel: Repeated high-frequency stimulation (HFS; indicated here by three arrows) in synaptic input S1 that induces input-specific L-LTP triggers two synaptic events at the activated postsynaptic neuron. One is the setting of a synapse-specific synaptic tag that marks the activated synapse, the other is the synthesis of synapse-unspecific (in the soma or dendrites) plasticity-related products (PRPs) which are diffusely distributed though the neuron. The synaptic tag functions to sequester or interact with PRPs, thereby stabilizing synaptic strength of the activated synapse. **Right panel**: If a nearby synapse of the same postsynaptic neuron receives one single train of HFS (indicated here by one arrow) in synaptic input S2 (within 1 h after L-LTP in S1) that normally induces E-LTP, a synaptic tag can be set to mark the synapse but no PRPs are synthesized. However, synapse-unspecific PRPs synthesized by L-LTP of S1 can be shared, allowing the capture of PRPs by the tagged synapse and thus the transformation of E-LTP into L-LTP in S2.(Source: Adapted from Frey and Morris, 1998b; Sajikumar and Frey, 2004b).

Not only STC was demonstrated at the rodent hippocampus, but also later STC was confirmed in the invertebrate animal of *Aplysia* neurons (Martin et al., 1997). In the

cultured systems of *Aplysia* neurons, a single bifurcated *Aplysia* sensory neuron forms synaptic contacts with two spatially separated motor neurons, a simplified model to study sensitization in *Aplysia*. Application of one single puff of serotonin (5-HT) to one axon branch gives rise to a short-term facilitation (STF) that lasts for minutes and involves covalent modification of preexisting proteins, whereas delivery of five puffs of 5-HT produces a long-term facilitation (LTF) that involves transcription, translation, and the growth of new synaptic connections. Similarly, a single puff of 5-HT delivered to one axonal branch can be facilitated into LTF if five puffs of 5-HT are applied to the other axonal branch within a discrete time window, showing STC. These observations in rodent of rat and invertebrate of *Aplysia* further indicate an associative property of memory formation is evolutionally conserved (Martin and Kosik, 2002).

Intriguingly, STC is not limited to LTP, NMDAR-dependent LTD at the Schaffer collateral-CA1 synapses also shows STC. This was revealed by the findings that either transient protein synthesis independent E-LTD or L-LTD that depends on protein and mRNA- synthesis but in the presence of protein synthesis inhibitors can be converted into enduring L-LTD when the stimulus that induces L-LTD is applied to another separated synaptic input in the same population of neurons (Kauderer and Kandel, 2000; Sajikumar and Frey, 2004b).

Strikingly, Shires et al., in 2012 could for the first time show that STC also exists in the hippocampus of living rat. This is supported by the evidences that decaying LTP (either E-LTP or L-LTP in the presence of protein synthesis inhibitor) of the ipsilateral Schaffer-collateral input to CA1 can be stabilized into an enduring L-LTP, by prior or subsequent strong HFS of an independent contralateral commissural input to a common population of CA1 neurons (Shires et al., 2012). Additional supportive and indirect evidences of STC *in vivo* are that LTP persistence in the free moving rodents can be reinforced if the animal has been exposed to appetitive behavioral stimuli (such as unexpected novelty or water supply to thirsty rats) that upregulates the availability of PRPs (Seidenbecher et al., 1997; Kemp and Manahan-Vaughan, 2004; Ballarini et al., 2009; Wang et al., 2010b; Redondo and Morris, 2011).

Collectively, the STC hypothesis explains how short-term plasticity (E-LTP/E-LTD) can be transformed into long-term plasticity (L-LTP/L-LTD). Since the temporal persistence of LTP/LTD is proportional to the persistence of memory (Barnes and McNaughton, 1985), the widespread STC phenomenon provides a conceptual basis for how weak or short-term

memory (STM) can be stabilized to long-term memory (LTM) and thus underlies memory consolidation at a cellular level (Redondo and Morris, 2011). Different with the standard model of memory consolidation in which the persistence of memory relies heavily on the characteristics of neural stimuli (such as the strength and repetition) at the time of memory encoding (McGaugh, 2000), the STC hypothesis implies that neural events that occur before or immediately after memory encoding in the relevant neural network also determines the persistence of memory (Redondo and Morris, 2011). Additionally, it explains why the inconsequential events that occur in association with novelty or "flashbulb memories" (such as the terrorist attacks of September 11th, 2001) can be remembered for a longer time (and may persist into LTM) than it would otherwise be, underlying associative forms of LTM (Frey and Morris, 1997, 1998b; Redondo and Morris, 2011).

1.2.2 Cross-Capture

The STC model has been expanded to include heterosynaptic interactions between LTP and LTD, a paradoxical phenomenon referred as "cross-capture" (Sajikumar and Frey, 2004b) (Figure 1. 8). Cross-capture shows that NMDAR-dependent L-LTP/L-LTD in one synaptic input is capable of transforming the opposite, protein synthesis-independent E-LTD/E-LTP in a second synaptic input of the same population of neuron into its long-lasting form. This striking finding further suggests that either L-LTP or L-LTD induction could lead to a common set of proteins support the opposing process, whereas distinct synaptic tags that allow recruitment of distinct subsets of proteins would determine whether that common proteins lead to the persistence of LTD or LTP in a given synapse (Kelleher et al., 2004). It also provides a cellular basis that explains how bidirectional changes in synaptic strength can be stabilized simultaneously (Reymann and Frey, 2007).

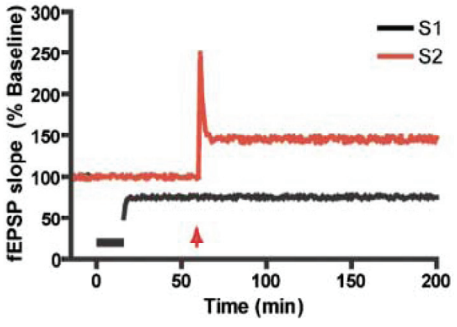


Figure 1. 8 | Cross-capture.
A single tetanic train used to induce E-LTP is delivered to input 2 (S2) in close temporal proximity to strong low frequency stimulation (LFS) that produces an L-LTD in S1, here E-LTP in S2 is converted to L-LTP that would otherwise go to baseline within one hour, suggesting E-LTP creates a synaptic tag that can capture the gene expression products from persistent L-LTD, thereby leading to enhanced potentiation. The bar represents delivery of LFS. (Source: Kelleher et al., 2004).

1.2.3 Inverse Synaptic Tagging

Maintenance of synaptic weight contrast between strong and weak synapses within active neurons is essential for securing the consolidation of input-specific L-LTP and memory engram. Whereas active synapses following a strong synaptic stimulus are marked for synaptic strengthening by STC process (Redondo and Morris, 2011), it remains elusive how the inactive synapses in potentiated neurons are marked for synaptic weakening. Recently, Okuno et al reported that after strong neural activity Arc, an immediate early gene, is rapidly upregulated and specifically anchored at inactive synapses where it interacts with the inactive form of CaMKIIβ (β-isoform of CaMKII), contributing to AMPARs endocytosis at the inactive synapses (Okuno et al., 2012). These observations were referred to as "inverse synaptic tagging" model (Figure 1. 9) in which the inactive synapses at potentiated neurons are marked by the "inverse synaptic tag" – inactive form of CaMKIIβ that directs the negative plasticity factor such as Arc to the weak synaptic sites (Okuno et al., 2012; Nonaka et al., 2014). The inverse synaptic tagging provides a cellular mechanism that explains how the undesired enhancement of weak synapses in potentiated neurons is prevented, thereby securing the difference of synaptic weight between strong and weak synapses overtime (Okuno et al., 2012; Whalley, 2012; Nonaka et al., 2014). The coexistence of synaptic tagging and inverse synaptic tagging in spines may allow synapses to use a two-bit tagging code for maintaining input-specific memory engrams over time (Nonaka et al., 2014).

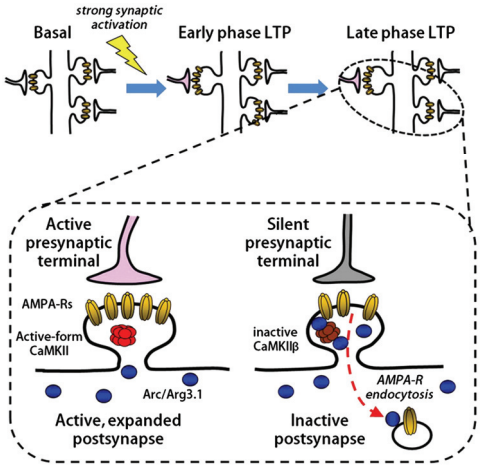


Figure 1. 9 | Inverse synaptic tagging model. After strong synaptic activation that leads to L-LTP induction, more AMPARs are inserted into the PSD of synapses that receive strong stimuli, whereas Arc is synthesized rapidly in a cell wide manner. During the late phase of LTP, the synaptic localization of Arc is differently regulated by the history of synaptic activity. In the synapses that received strong synaptic activation for L-LTP induction, CaMKIIβ is activated and its interaction with Arc is limited. In contrast, synapses with low activity (inactive synapses) contain an inactive form of CaMKIIβ (acts as part of an "inverse synaptic tag") which provides a scaffold for Arc (as negative plasticity factors) at the synapse, thereby Arc is specifically targeted to inactive synapses. As a result, AMPARs at the PSD of the inactive synapses are removed, maintaining synaptic weakening. (Source: Okuno et al., 2012).

1.2.4 The Nature of Synaptic Tag

Although a full understanding of the molecular and cellular mechanisms underlying the synaptic tag setting is still lacking, there are plenty of evidences showing that candidate mechanisms for synaptic tagging should at least satisfy the following criteria (reviewed by Martin and Kosik, 2002; Kelleher et al., 2004; Redondo and Morris, 2011): 1) a tag should be spatially restricted to the activated synapses; 2) the lifetime of a tag is transient lasting 1-2 h; 3) the activation of a tag does not need protein synthesis; 4) a tag needs to interact with cell wide PRPs to facilitate synaptic capture; 5) a tag should be process-specific, i.e., distinct tags are created in response to LTP and LTD induction. As such, a number of possible postsynaptic modifications following plasticity induction, including phosphorylation or dephosphorylation events at the stimulated synapses (Sajikumar et al., 2007; Redondo et al., 2010), changes in the actin microfilament network (Ramachandran and Frey, 2009; Bosch et al., 2014), compartmentalized mRNA translation (Wang et al., 2010a), proteasome mediated protein degradation (Cai et al., 2010) and TrkB activation (Lu et al., 2011), have been proposed as appropriate candidates for the synaptic tag. Hence, synaptic tag is not simply a single molecule, but rather it should be considered as "a state of the synapse" that involves local molecular changes at the activated synapses that marks the synaptic events having occurred (Redondo and Morris, 2011).

Tag Setting Mediated by Kinase

Specific phosphorylation or dephosphorylation events at the stimulated synapses that associates with LTP or LTD induction meet several of the criteria for a tag, as they enable a synapse to ''remember'' the previous synaptic events happened only at the activated synapses (Martin and Kosik, 2002). As such, persistently active kinases such as CaMKII, PKA and PKMζ have been proposed as suitable candidates for the tagging machinery in LTP (Martin and Kosik, 2002; Reymann and Frey, 2007; Redondo and Morris, 2011), whereas extracellular signal-regulated kinase 1/2 (ERK1/2) mediates LTD-specific tag (Sajikumar et al., 2007).

CaMKII, which becomes autonomously and persistently active by autophosphorylation, is a well established kinase that mediates the synaptic tagging during LTP (Sajikumar et al., 2007; Redondo et al., 2010; Redondo and Morris, 2011). Once activated by elevation of Ca^{2+} during LTP induction, it translocates specifically from the cytoplasm to the activated synapse by simple diffusion or driven by spine enlargement (Lisman et al., 2012). Using an elegant optical method by which spatiotemporal dynamics of CaMKII activation could be

monitored, Lee et al., demonstrated directly that CaMKII is activated within seconds after the start of synaptic stimulation and the activation is restricted to the stimulated spine (Lee et al., 2009) – a property perfectly meets the criteria of a synaptic tag. In addition, electrophysiological evidences show although pharmacological inhibition of CaMKII leaves the functional expression of E-LTP unaffected, do inhibit its ability to capture PRPs, indicating CaMKII activation is crucial in initiating the tag setting process (Reymann and Frey, 2007; Sajikumar et al., 2007; Redondo et al., 2010). Moreover, CaMKII plays a structural role by regulating the actin cytoskeleton (Okamoto et al., 2007), which is also essential for the synaptic tag setting (Okamoto et al., 2009). Remarkably, CaMKII mediates the learning tag setting in behavioral tagging during memory formation (Moncada et al., 2011). Consistently, CaMKII activation was demonstrated to be both necessary for LTP and LTM (Lisman et al., 2002; Lisman et al., 2012), as mutated CaMKII mice showed impaired LTP and profoundly LTM deficits (Silva et al., 1992; Giese et al., 1998).

In *Aplysia*, the initiation of synapse-specific plasticity (i.e., the ability of a single pulse of 5-HT to capture LTF) depends on a covalent cAMP-dependent PKA mediated component, thus PKA is considered as a mediator for the tag setting process during LTF in *Aplysia* (Casadio et al., 1999).

Synaptic Tag Duration and Resetting

The synaptic tag is only transiently active, with a lifetime of approximately 60 min (Martin and Kosik, 2002; Redondo and Morris, 2011). This is revealed by "weak-before-strong" brain slices protocols at the Schaffer collateral pathway-CA1 synapses with the key evidences that the expression of E-LTP following a weak tetanus in one pathway can be stabilized into L-LTP only if the weak stimulation was delivered 60 min before inducing L-LTP in the second pathway, but not when L-LTP induction was delayed 2 or 4 h (Frey and Morris, 1998a). Similarly in *Aplysia* sensory-motor synapses *in vitro*, capture of LTF was possible only when the single puff of 5-HT that produce STP was applied either simultaneously or 1-2 h before the five puffs of 5-HT are applied to the other connection (Casadio et al., 1999).

The duration of the synaptic tag is not fixed but subject to regulatory mechanisms that can accelerate or delay the turnover of synaptic tags (Barco et al., 2008). Synaptic tag may be inactivated *passively* by some underlying biological process such as degradation or dephosphorylation. Alternatively, it can be reset or delete *actively* in an activity-dependent manner by LFS that induces depotentiation (DP) of LTP (Sajikumar and Frey, 2004a;

Young and Nguyen, 2005). Of note, the effective time window for resetting the synaptic tag is less than 10 min after the initial induction of E-LTP (Sajikumar and Frey, 2004a). Tag resetting by DP may be mediated by the activation of protein phosphatases which counteract the action of the kinase activities involved in setting the tag (Barco et al., 2002). Functionally, tag resetting prevents the creation of a memory trace formation and therefore may underlie the process of forgetting (Sajikumar and Frey, 2004a).

Synaptic Tag Setting is Compartment-Specific

Although synaptic tagging occurs at both the CA1 subfield of stratum radiatum and the stratum oriens and requires the same physiological tagging process (one train of tetanic stimulation), capture across the two compartments follows different rules and needs a stronger stimulation (two trains of tetanic stimulation) than capture within a compartment (Alarcon et al., 2006), indicating synaptic tag setting is specific to a compartment either apical or basilar. Indeed, protein kinases differentially mediate the tag setting process in the apical and basal dendrites. For instance, CaMKII mediates LTP specific tags in apical dendrites, whereas in the basal dendrites, CaMKII is unimportant for LTP tag setting and both PKA and PKMζ are necessary (Sajikumar et al., 2007). However, it is noteworthy that PKA activation has been implicated in tagging of LTP in apical dendrites also (Barco et al., 2002; Young and Nguyen, 2005; Alarcon et al., 2006). The existence of compartment-specific tags may contribute to enhance the efficacy of synaptic tags without involving complicated pathways of intracellular trafficking and thus may increase the efficiency of protein capture that otherwise would be degraded without use (Alarcon et al., 2006).

1.2.5 The Identity of Plasticity-Related Proteins

STC reveals a principle of "sharing" potentiation, this is due to that either enduring forms of LTP or LTD could produce a pool of plasticity-related products (PRPs) cell wide which can be captured by the tagged synapses nearby. The PRPs function to prolong potentiation not only at strongly tetanized pathway, but also at independent but convergent, weakly tetanized pathways if synaptic tags were set. In addition, capture of PRPs is critical for the stabilization of structural alterations to a dendritic spine (Redondo and Morris, 2011). It is important to note that the PRPs induced by either L-LTP or L-LTD induction are mutually overlapping as according to cross-capture, L-LTP/L-LTD has the ability to support the opposing process E-LTD/E-LTP (Sajikumar and Frey, 2004b). Up to date, the molecular

identity of all the PRPs is still unknown, but includes PKMζ, BDNF (brain-derived neurotrophic factor), GluR1, Homer1a, and Arc (reviewed by Martin and Kosik, 2002; Redondo and Morris, 2011).

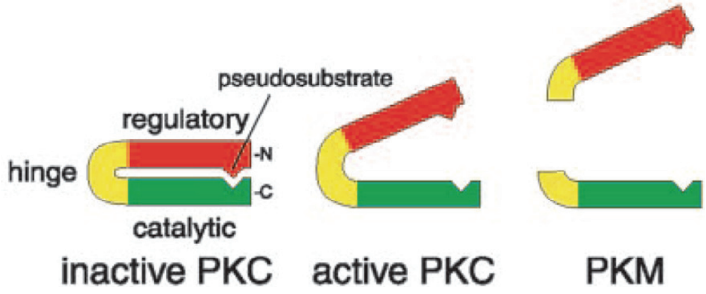


Figure 1. 10 | Model of PKC and PKMζ structure.
Details see text 1.2.5. PKC, protein kinase C; PKM, protein kinase M. (Source: Hernandez et al., 2003).

PKMζ's ability to act as a PRP is due to its unique structure as an autonomously active fragment of PKC (Figure 1. 10). Most full-length PKCs consist of both an amino-terminal regulatory domain and a carboxy-terminal catalytic domain. The regulatory domain contains second messenger-binding sites and an autoinhibitory *pseudosubstrate*, which interacts with and inhibits the catalytic domain. PKMζ lacks the regulatory domain, thus once activated, it is constitutively active (Hernandez et al., 2003). Although other constitutively activated protein kinases like CaMKII, PKA and PKC could also persistently potentiate synapses to maintain LTP, they are important only in LTP induction instead of for maintenance (Malinow et al., 1988; Otmakhov et al., 1997). PKMζ is the first identified LTP-specific PRP and is critical for the transformation of E-LTP into L-LTP during the process of both STC and cross-capture (Sajikumar et al., 2005a; Sajikumar et al., 2009; Sajikumar and Korte, 2011a). This is supported by the key evidences that 1) PKMζ is synthesized over tens of minutes following L-LTP but not E-LTP induction (Sacktor et al., 1993; Hernandez et al., 2003); 2) Postsynaptic diffusion of PKMζ produces a gradual and persistent synaptic potentiation which occludes the subsequent induction of LTP (Ling et al., 2002); 3) Application of PKMζ inhibitor ZIP has no effect on E-LTP, but reverses the potentiation of L-LTP (Ling et al., 2002; Sajikumar et al., 2005b; Serrano et al., 2005) and storage of spatial information (Pastalkova et al., 2006); 4) During STC, application of ZIP prevents not only the capture or facilitation of E-LTP in one synaptic input but also L-LTP expression in the second synaptic input (Sajikumar et al., 2005b); 5) In cross-capture, ZIP has no effect on L-LTD but could nonetheless reverse L-LTP (Sajikumar et al., 2005b).

But how is PKMζ activity maintained due to the constant turnover of individual PKMζ molecules? This is mainly due to that PKMζ is synthesized by a positive feedback loop (reviewed by Sacktor, 2011), see also Figure 1. 11). At basal state, PKMζ mRNA translation is suppressed by the action of PIN1 (protein interacting with NIMA1). Following LTP induction, several signaling pathways activated by Ca^{2+} entry through NMDAR decrease PIN1 activity, allowing PKMζ synthesis. After its synthesis, PKMζ phosphorylates and inhibits the activity of PIN1, thereby sustaining PKMζ synthesis. Through this, the local translation of PKMζ could be continuously translated at the dendrites, maintaining high levels of it at the strengthened synapses. Accordingly, PKMζ has been proved to be not involved in STM, but rather is a necessity that stores LTM at least 3-month after memory encoding (Shema et al., 2009).

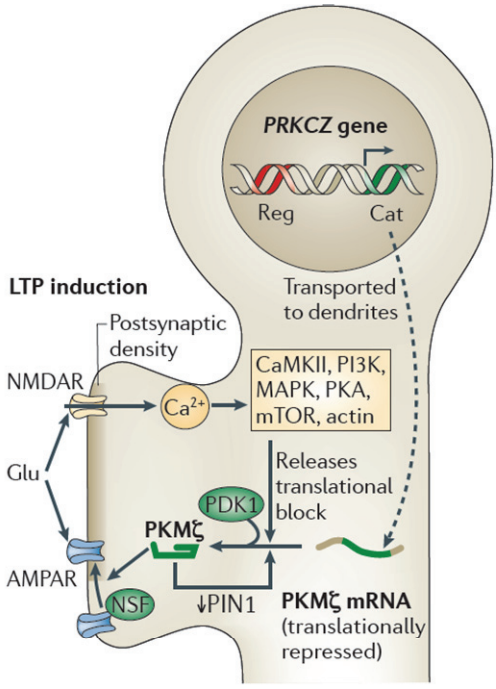


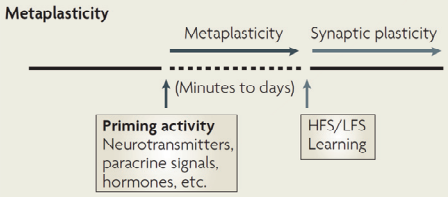
Figure 1. 11 | PKMζ formation in LTP.
PKMζ mRNA, which encodes a PKCζ catalytic domain (Cat; shown in green) without a regulatory domain (Reg; shown in red), is generated by an internal promoter within the protein kinase C zeta (PRKCZ) gene. The PKMζ mRNA is transported from the nucleus to the dendrites of neurons. Under basal conditions it is translationally repressed. During LTP induction, multiple signalling pathways stimulated by NMDAR activation cause the release of translational block. The newly translated PKMζ is rapidly phosphorylated by phosphoinositide-dependent protein kinase 1 (PDK1), which increases the constitutive kinase activity of PKMζ. PKMζ enhances its own translation by phosphorylating (protein interacting with NIMA1 (PIN1). The persistent activity of PKMζ then maintains increases in postsynaptic AMPARs through an N-ethylmaleimide-sensitive factor (NSF)-dependent pathway. CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; Glu, glutamate; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A. (Source: Sacktor, 2011).

1.3 Metaplasticity

Optical function of neural networks depends on the interplay between Hebbian plasticity and homeostatic regulation. Homeostatic modulation occurs either *concurrently* with synaptic plasticity by intracellular signaling molecules such as GABA (γ-aminobutyric acid), cytokines and hormones, or *previously* by neural activity that alters the threshold of synaptic plasticity. This activity dependent regulation of synaptic plasticity by prior neural activity is referred as metaplasticity, a term coined by W.C. Abraham and M.F. Bear in 1996 to encompass a plethora of phenomena (Abraham and Bear, 1996). The prefix "meta-"of the term implies it is a high level form of plasticity in nature. Shortly, metaplasticity is the "plasticity of synaptic plasticity"(Abraham and Bear, 1996).

Box 1 | Concept of metaplasticity.

Metaplasticity is the modulation of synaptic plasticity by prior neural activity (priming). The priming signal can entail electrical stimulation of neural activity, pharmacological activation of specific transmitter, etc. Of note, metaplasticity can occurs in the absence of changes in the excitability of the stimulated neurons/network per se. Nevertheless, essentially, it entails a change in the physiological or biochemical state of neurons or synapses that alters their ability to generate synaptic plasticity. (Source: Abraham, 2008).



1.3.1 Basic Concepts

The basic idea of metaplasticity is that the induction threshold of synaptic plasticity is not static but instead varies dramatically according to the recent history of neural activity (Figure 1. 12), i.e., synapse's previous history of activity determines its current plasticity (Abraham and Bear, 1996; Abraham and Tate, 1997). Prior neural activity by NMDAR activation, for example, raises the threshold for LTP induction, therefore inhibiting the subsequent induced LTP. In contrast, pre-stimulation of group 1 metabotropic glutamate receptor (mGluR) lowers the threshold for LTP induction, thus facilitating both the induction and persistence of the subsequent LTP. Intriguingly, this sliding threshold of synaptic plasticity was also predicted by the BCM computational model (Bienenstock et al., 1982). As the first instantiation of the metaplasticity concept, the BCM theoretical model incorporates a modification threshold (θm) of synaptic plasticity to provide stability, i.e., θm is not static but dynamically varies in a bidirectional manner with the history of integrated prior postsynaptic firing. Specifically, θm decreases at low levels of previous postsynaptic activity, favoring LTP induction over LTD. In contrast, θm increases if the

recent postsynaptic activity is maintained at high levels, therefore making LTP harder to obtain and LTD easier to get (Bienenstock et al., 1982; Cooper and Bear, 2012).

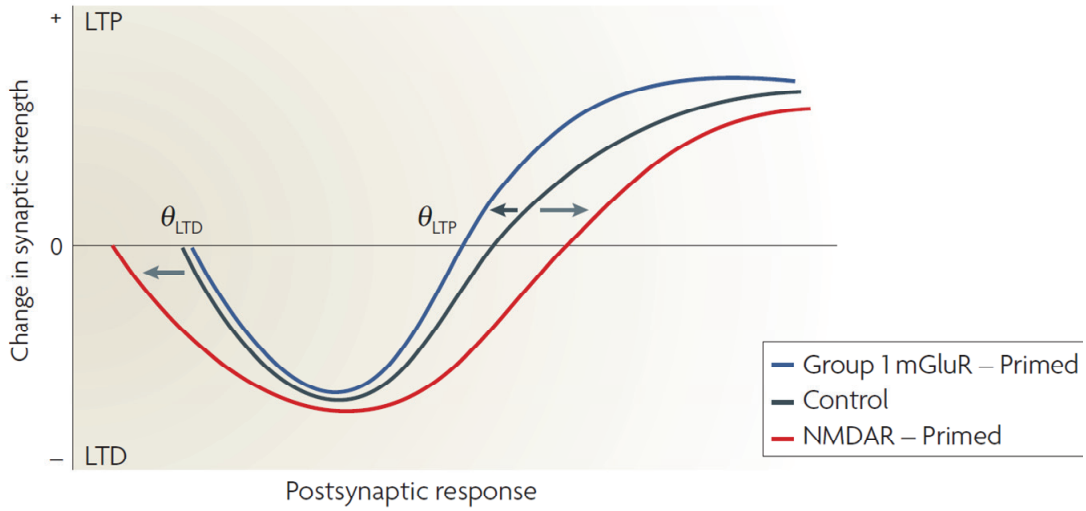


Figure 1. 12 | Glutamate-receptor-mediated metaplasticity.
In unprimed conditions, synaptic strength changes in response to afferent activity at different levels of postsynaptic cell firing at excitatory synapses on hippocampal CA1 pyramidal cells (black line). θLTD and θLTP represent the threshold level of postsynaptic firing that is required in order for afferent stimulation to result in LTD or LTP, respectively. Prior glutamate receptor activation (priming) could influence the subsequent LTP and LTD. NMDAR activation (red line) lowers the threshold for LTD (θLTD) while raising the threshold for LTP (θLTP), as indicated by the gray arrow. By contrast, group 1 mGluR activation (blue line) lowers θLTP, as shown by the black arrow. (Source: Abraham, 2008).

Metaplasticity states can be expressed over a broad range of *spatial extents* of the postsynaptic cell (space) and distinct *time scales* (time) (reviewed by Abraham, 2008; Hulme et al., 2013; Muller-Dahlhaus and Ziemann, 2014). Metaplasticity can be input-specific (*homosynaptic metaplasticity*), which refers to that the synapses that show altered plasticity is confined to the synapses that have participated in the priming neural activity. Metaplasticity also spread to additional synapses that did not participate in the initial prior neural activity, either globally in the cell wide or confined to neighboring synapses on the dendritic compartment, a phenomenon termed as *heterosynaptic metaplasticity*. Homosynaptic metaplasticity can be elicited by glutamate receptor activation such as NMDARs and mGluRs, while heterosynaptic metaplasticity results from changes in cell excitability, intercellular communication mediated by astrocytes, and modulation of STC by PRPs mechanisms. Behaviorally, activation of homosynaptic metaplasticity would be well placed to prepare specific synapses to encode specific content, while initiation of heterosynaptic metaplasticity may increase general preparedness for learning. The duration of metaplasticity state can be expressed over distinct time scales,

from minutes to days and even weeks. Thus, metaplasticity is a mechanism that integrates synaptic events across space and time (Abraham, 1999).

1.3.2 mGluR-Mediated Metaplasticity

Prior activation of group 1 mGluR, either by synaptically released glutamate or its agonist DHPG [(R,S)-3, 5-dihydroxyphenylglycine], sets up a metaplastic state such that both the induction and persistence of the subsequent LTP is facilitated, a phenomenon best studied in CA1 *in vitro* (Abraham and Bear, 1996; Abraham and Tate, 1997; Abraham, 2008). Two mechanisms have been suggested to mediate this effect of increasing LTP induction. In the first, mGluR activation inhibits Ca^{2+}-activated K^{+} current that mediates the slow afterhyperpolarization (sAHP), leading to enhanced spike discharges during LTP-inducing tetanic stimulation (Cohen et al., 1999; Ireland et al., 2004). Secondly, mGluR activation couples to phospholipase C (PLC) (Cohen et al., 1998) which leads to activation of PKA that phosphorylates Ser845 of GluR1, resulting in increased trafficking of AMPARs to the extrasynaptic membrane (Oh et al., 2006). Prior mGluRs activation enhances the persistence of LTP as well. The key evidences are that HFS of mGluRs sets a "molecular switch" which negates the need for their activation during subsequent LTP induction, thus lowering the stimulus requirements for plasticity (Bortolotto et al., 1994). Similarly, pharmacological or synaptic activation of mGluRs primes LTP, transforming a decaying form of LTP into a longer lasting form (Cohen et al., 1998; Raymond et al., 2000). Prior mGluR activation enhances LTP persistence by synaptodendritic synthesis of PRPs (Raymond et al., 2000; Sajikumar and Korte, 2011a). This is mediated by the activation of PLC and the subsequent Ca^{2+} from intracellular stores, as well as the entry of Ca^{2+} from store-operated channels (SOCs) in the plasma membrane (Mellentin et al., 2007). These processes in turn lead to the activation of several kinases such as ERK1, ERK2, PKC and αCaMKII. PKMζ recently has been reported as one of the PRPs that generated by mGluRs activation (Sajikumar and Korte, 2011a).

Of note, in dentate gyrus, prior activation of group 1 or group 2 mGluRs by HFS at medial perforant path synapses inhibits subsequent LTP by activating PKC and p38 MAPK (mitogen-activated protein kinase) mechanisms, an opposite effect as that of CA1 (Gisabella et al., 2003). Thus, mGluR mediated metaplasticity contributes to LTP distinctly, with the effect varies depending on the neural circuit.

1.3.3 RyR-Mediated Metaplasticity

Ryanodine receptors (RyRs; named after the plant alkaloid ryanodine) are a class of intracellular Ca^{2+} channels found in various forms of excitable animal tissue such as muscles and neurons. RyRs are located on the intracellular endoplasmic reticulum (ER) membrane. It is a ligand-gated Ca^{2+} channel, which can be activated by its agonist including Ca^{2+} itself, leading to channel opening and Ca^{2+} release, a process known as Ca^{2+}-induced Ca^{2+}-release (CICR) (Miller, 1991; Zucchi and Ronca-Testoni, 1997). Thus, RyRs could amplify the activity-dependent Ca^{2+} influx. RyRs exist in three isoforms (RyR1-3) and are widely distributed in mammalian brain. In mature hippocampal CA1, RyRs are densely present throughout the pyramidal neurons, including the layers of stratum oriens, stratum pyramidale and stratum radiatum, but are absent at the stratum lacunosum-moleculare (Hertle and Yeckel, 2007).

In a functional perspective, RyRs regulate brain function in a broad range such as protein synthesis (Paschen et al., 1996), neuronal excitability (Verkhratsky, 2005), neurotransmitter release (Mothet et al., 1998; Bouchard et al., 2003), action potential hyperpolarization (Kawai and Watanabe, 1989), and fast axonal transport (Breuer et al., 1992). Strikingly, RyRs activation contributes to hippocampal synaptic plasticity and most likely to LTP induced by weak protocols (Obenaus et al., 1989; Harvey and Collingridge, 1992; Behnisch and Reymann, 1995; Wang et al., 1996; Balschun et al., 1999; Szinyei et al., 1999; Raymond and Redman, 2002), although its role in L-LTP was also suggested (Lu and Hawkins, 2002). Supportive evidences are that application of ryanodine receptor (RyR) antagonist inhibits the induction of hippocampal LTP (Behnisch and Reymann, 1995; Raymond and Redman, 2002), while its agonist could facilitate LTP (Lu and Hawkins, 2002; Mellentin et al., 2007; Sajikumar et al., 2009; Grigoryan et al., 2012). Of note, this reinforcing effect is more prominent in the ventral hippocampus perhaps due to a higher distribution of RyRs in ventral hippocampus than in the dorsal part (Grigoryan et al., 2012). Furthermore, RyR activation is involved in CREB-mediated gene expression (Hardingham et al., 2001), spatial learning (Balschun et al., 1999) and memory (Zhao et al., 2000; Edwards and Rickard, 2006; Galeotti et al., 2008; Baker et al., 2010; Adasme et al., 2011). In turn, blockade of RyR activity in the brain decreased both memory formation and retrieval (Edwards and Rickard, 2006) and even causes amnesia in animals (Galeotti et al., 2008). In particular, RyR3 deletion in mice showed impaired CaMKII activation, E-LTP expression, spatial learning and contextual fear conditioning (Balschun et al., 1999; Kouzu et al., 2000). And, both RyR2 mRNA and proteins were significantly increased in the

hippocampus of rats trained in an intensive water maze task (Zhao et al., 2000).

RyR-mediated metaplasticity has been reported to facilitate LTP in the hippocampus *in vitro* (Mellentin et al., 2007; Sajikumar et al., 2009). Initially, RyRs were found to act downstream of group 1 mGluR mediated priming of LTP. This is supported by the evidences that RyRs are involved in the group 1 mGluR activated Ca^{2+} mobilization from intracellular stores (Nakamura et al., 2000; Morikawa et al., 2003; Tozzi et al., 2003), and blockade of RyRs prevents DHPG priming of LTP (Mellentin et al., 2007). Nevertheless, later it was shown that direct activation of RyRs with its agonist RYA (10 μM) is sufficient to prime the subsequent LTP, an effect that is as effective as that of the group 1 mGluR priming of LTP (Mellentin et al., 2007; Sajikumar et al., 2009). Moreover, RyRs activation by its agonist RYA or caffeine also facilitates STP, and most intriguingly enables the synaptic tag setting (Sajikumar et al., 2009).

1.4 Alzheimer's Disease and Synaptic Plasticity

Alzheimer's disease (AD), firstly described by the German neuropathologist Alois Alzheimer in 1906 (Alzheimer, 1907), is the most common form of age-related neurodegenerative disorder and the leading cause of dementia among the elderly. AD is featured with progressive memory loss. In the early clinical phase, AD is characterized with a remarkably pure impairment of declarative memory of recent events (Bäckman et al., 2001). As the disease progresses, LTM loss and cognitive dysfunction in other domains that interfere with learning, language, orientation, recognition, and judgment occur. The late stage of AD is featured with severe global impairment of cognitive function, leading to the loss of the abilities to do basic daily tasks such as speech and eating. Age is a major risk factor for AD, with the prevalence rate rising dramatically with age (Brookmeyer et al., 1998). Due to increased life expectancy, AD is predicted to effect more than 115 million people worldwide by 2050 (Brookmeyer et al., 2007). So far, there is no effective treatment for this disease, which worsens as it develops, ultimately leading to death.

1.4.1 Alzheimer's Disease

The pathological hallmarks of AD, as described by Alois Alzheimer in 1907, are the formation of extracellular amyloid plaques, intracellular neurofibrillary tangles (NFTs) and extensive neuronal loss (Goedert and Spillantini, 2006). NFTs deposit within neurons and

are composed predominantly of hyperphosphoryated, aggregated form of the microtubule binding protein tau, whereas the amyloid plaques are accumulations of molecules in the extracellular space which are made of the small protein (4 kDa) called amyloid β-peptide (Aβ), both of which could be found in the neocortex and limbic brain regions, such as the hippocampus and amygdale.

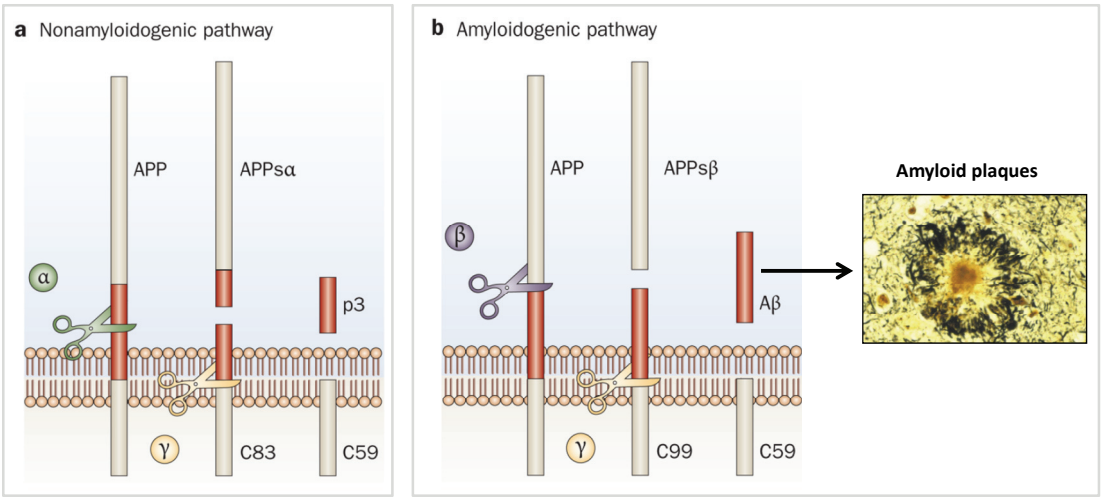


Figure 1. 13 | APP processing and its products.
The transmembrane protein APP can be processed by two main pathways: non-amyloidogenic pathway and amyloidogenic pathway. **(a)** The non-amyloidogenic APP processing pathway involves proteolytic cleavages by α- and γ-secretases, which results in the generation of a large APPsα and carboxyl terminal fragments including P3 and C59. **(b)** The amyloidogenic pathway releases Aβ peptides through β- and γ-secretases cleavage. Aβ could auto-aggarate and further deposit to form amyloid plaques. Aβ, amyloid-β; APP, amyloid precursor protein; APPsα, soluble amyloid precursor protein-α; APPsβ, soluble amyloid precursor protein-β; C83, carboxy-terminal fragment 83; C59, carboxy-terminal fragment 59; C99, carboxy-terminal fragment 99. (Source: Sisodia and St George-Hyslop, 2002; De Strooper et al., 2010).

Most Aβ is generated through proteolytic cleavage of the much larger protein amyloid precursor protein (APP) by the β-and γ-secretase that give rise to the N terminus and C terminus of Aβ, respectively (Figure 1. 13) (Goedert and Spillantini, 2006). Three principal forms of Aβ that comprise 38, 40 or 42 amino acid residues are produced from APP. Among them, Aβ42 is far more prone to oligomerize and form amyloid fibrils than the Aβ40 peptide (Burdick et al., 1992; Jarrett et al., 1993). After its cleavage, Aβ is released into the extracellular space (Hardy and Selkoe, 2002) and is a natural product that is present in the brains and cerebrospinal fluid (CSF) of normal animals throughout life (Haass et al., 1992; Seubert et al., 1992). At lower concentration and in monomeric form, Aβ plays a physiological role. This is supported by the findings that endogenous Aβ42 is critical in synaptic plasticity and memory (Puzzo et al., 2011), and on the other hand, low picomolar amounts of exogenously applied Aβ42 even enhances synaptic plasticity and

memory (Puzzo et al., 2008). However, if above a certain critical concentration, Aβ can self-associate to form several different assembly forms, from Aβ monomers to soluble oligomers, protofibrils, and fibrils, which aggregate to form plaques (Lansbury, 1992, 1999). In particular, oligomeric forms of Aβ are the principle mediators of synapse loss and neuronal injury (Kayed et al., 2003; Walsh and Selkoe, 2007).

Aβ instead of amyloid plaques has been the focus of AD research. It is considered to be the primary influence driving AD pathogenesis (Hardy and Selkoe, 2002) and a better measure in the onset of cognitive dysfunction (Oddo et al., 2003; Billings et al., 2005). This is revealed by the evidences that cognitive dysfunction in AD transgenic mice with increased Aβ occurs prior to the signs of amyloid deposition (Mucke et al., 2000; Billings et al., 2005). Additionally, although synapse loss is most prominent in the vicinity of plaques which may be a reservoir of synaptotoxic Aβ (Moolman et al., 2004; Tsai et al., 2004), it occurs in regions in advance or independent of plaque formation (Hsia et al., 1999; Buttini et al., 2002; Wu et al., 2004). Consistently, amyloid plaque density in the human brain does not correlate well with the severity of dementia (Katzman, 1986; Terry et al., 1991). Nowadays, various genetic manipulations of familial Alzheimer's disease (FAD) genes including APP, presenilin-1 and -2 (PS1 and PS2) (Chartier-Harlin et al, 1991; Goate et al, 1991; Sherrington et al, 1995) that enhance the concentration of toxic Aβ42 (Martin et al., 1995; Scheuner et al., 1996; Qi et al., 2003; Billings et al., 2005) are used as effective model systems for the investigations of AD.

1.4.2 Synaptic Plasticity Deficits in Alzheimer's Disease

Mounting evidence suggests AD is primarily due to synaptic failure other than neuron loss as it begins with subtle alterations of synaptic efficacy before frank neuronal degeneration, and on the other hand neuronal loss itself does not account for the properties of the amnesia characteristic of AD (Small et al., 2001; Selkoe, 2002). Synaptic plasticity deficits, especially these in the layer II of the entorhinal cortex and the pyramidal layers of the hippocampus, has been demonstrated to be the best neurobiological correlate of the cognitive deficits in AD, suggesting that synaptic changes underlies the starting point of AD pathogenesis (Terry et al., 1991; DeKosky et al., 1996; Coleman and Yao, 2003). The neuropathological changes of AD which involves synaptic deficit are initially manifest at the entorhinal cortex, advance there to the hippocampus, and spread to the neocortex as it progresses (Braak and Braak, 1991). Since hippocampus and entorhinal cortex together form a network that is essential for the normal function of episodic memory, synaptic

changes in these two areas underlies the core and very early symptom of AD – episodic memory loss.

Hippocampal synaptic plasticity such as LTP and LTD are highly vulnerable to a rapid disruption by Aβ. A plethora of studies have shown that LTP both *in vitro* and *in vivo* can be blocked by direct exogenous Aβ application from distinct sources (e.g., synthetic, natural, and human AD-derived), whereas basal synaptic transmission is not affected (Cullen et al., 1997; Lambert et al., 1998; Freir et al., 2001; Klyubin et al., 2004; Shankar et al., 2008; Li et al., 2011). In close similarity, LTP is impaired in AD transgenic mouse models in which an abnormally high level of Aβ is present (Chapman et al., 1999; Trinchese et al., 2004; Ma et al., 2010). Of note, both LTP impairments and cognitive deficits could occur prior to the plaque formation in AD transgenic mice (Oddo et al., 2003; Gong et al., 2004; Jacobsen et al., 2006; Ma et al., 2010), which is consistent with the hypothesis that soluble Aβ oligomers, instead of amyloid plaques, in AD are synaptotoxic (Haass and Selkoe, 2007). Compared with LTP, fewer studies were performed to test the effects of Aβ on LTD. Several lines of reports lead to the general conclusion that Aβ oligomers facilitates low frequency stimulation (LFS)-induced LTD, an opposite effect as that of LTP. Direct evidence comes from the fact that acute exposure to synthetic Aβ fragments potently facilities the induction of hippocampal NMDAR-dependent LTD *in vivo* (Kim et al., 2001; Cheng et al., 2009). In close similarity, application of Aβ oligomers - derived from synthetic, cell culture, and human AD brains extracts - to the hippocampal slices facilitates the induction of CA1 LTD induced by a subthreshold LFS (Shankar et al., 2008; Li et al., 2009). However, there are reports showing that Aβ has no effects on hippocampal LTD that is induced by stronger LFS protocols (i.e., 900 pulses) (Wang et al., 2002; Raymond et al., 2003; Li et al., 2009). These synaptic plasticity deficits interfered by Aβ underlie cognitive deficits associated with AD (Selkoe, 2002; Walsh and Selkoe, 2004; Heneka et al., 2013).

1.5 Aim of Study

Metaplasticity governs different aspects of functional plasticity by integrating synaptic events across time (Hulme et al., 2013). Previous studies reveal that metaplasticity by RyR activation have substantial effects on functional plasticity in that it lowers the threshold for LTP induction, thus facilitating the subsequent LTP (Mellentin et al., 2007; Sajikumar et al., 2009). Strikingly, it lowers the threshold of subsequent STC by creating new synaptic tags (Sajikumar et al., 2009). The aim of my thesis is to investigate metaplasticity by RyR activation in regulating the functional plasticity in hippocampal neural networks, including not only physiological conditions but also pathological conditions of AD.

I. At the cellular basis, associative long-term memory are formed or maintained by the process of STC (Redondo and Morris, 2011). So far, due to the decay time course of the synaptic tag, STC in the hippocampus *in vitro* has been observed only for a limited time window of 1 h (Frey and Morris, 1998a). Nevertheless, association of weak memory can occur far beyond this period and its mechanism is not well understood. In my thesis, I investigated whether the time window of STC can be extended by processes of metaplasticity through prior RyR activation and if yes, what are the underlying mechanisms.

II. Synaptic plasticity deficits in the hippocampus are one of earliest events and the best neurobiological correlate of memory loss in the progression of AD (Selkoe, 2002). In the present study, synaptic plasticity, including L-LTP, its associativity process of STC, L-LTD and cross-capture were initially studied in the hippocampus of an AD mouse model – APP/PS1 mice (3-4-month old). The results show that L-LTP was impaired in APP/PS1 mice resembling that of an E-LTP. In addition, late associativity process of STC and cross-capture were absent in these transgenic mice. Motivated by the multiple function domains of metaplasticity, the present study address the question that whether inducing metaplasticity in the hippocampal synapses of this AD mouse model through prior RyR activation could prevent the synaptic plasticity deficits, and by this means restoring memory.

2 MATERIALS AND METHODS

2.1 Animals

Wistar Rats

In the first part of the thesis (i.e., the study of metaplasticity in the physiological conditions of hippocampus), male *Wistar rats* were used. As it has been reported that expression mechanisms of synaptic plasticity can be developmentally regulated (Dudek and Bear, 1993; Palmer et al., 2004), rats with an age of 5-7 weeks old which are young adult organisms with fully expressed adult receptor functions were used in the current study to avoid developmental problems (Sajikumar et al., 2005a).

APP/PS1 Mice

In the second part of the work (i.e., the study of metaplasticity in the pathological conditions of AD), *APP/PS1 mice* (3-4-month old) on the C57BL/6 background were used. Age-matched nontransgenic C57BL/6 mice were used as wild-type (WT) controls. The double transgenic APP/PS1 mouse is one of the most widely used transgenic mouse models for the investigation of AD (Radde et al., 2006). APP/PS1 mice used in the present study overexpress both human APP with Swedish double mutation (K670N and M671L) and human PS1 variant with delta E9 (PS1-dE9) deletion, both of which are well established to increase the production of toxic Aβ and later amyloid plaques formation (Puoliväli et al., 2002; Garcia-Alloza et al., 2006; Koffie et al., 2009). The APP/PS1 mice were created by co-injection of APPSwe and PS1dE9 vectors controlled by independent mouse prion protein (PrP) promoter elements, directing transgene expression to CNS neurons. It was reported that Aβ deposition in APP/PS1 mice starts at 6 weeks old in the cortex and 3-4 months old in the hippocampus (Radde et al., 2006). The unique characteristic of APP/PS1 mice is that they show fast appearance of phenotype and age-dependent decline of cognitive impairments (McGowan et al., 1999; Trinchese et al., 2004). However, the expression of protein synthesis-independent E-LTP is normal in this AD mouse model (3-4-month) (Figure 2. 1).

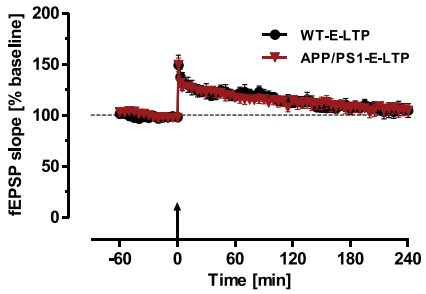


Figure 2. 1 | E-LTP in APP/PS1 mice

2.2 Hippocampal Slices Preparation

2.2.1 ACSF

The bathing media ACSF (artificial cerebral spinal fluid), which intends to mimic the actual physiological composition of the cerebral spinal fluid, was used during all the stages of slices preparation, incubation as well as electrophysiological recordings to keep the hippocampal slices alive and maintain neural activity. The composition of ACSF is very important in controlling the extracellular environments of the hippocampal slices and therefore has profound effects on the levels of subsequent neural activity. For instance, one study compared the varying Ca^{2+} and its effect on the potentiation of hippocampal LTP, and found that hippocampal LTP can achieve the maximal potentiation when the perfusion medium Ca^{2+} was at 2.5 mM (Dunwiddie and Lynch, 1979). The ionic composition of ACSF is generally similar among laboratories, however, there are minor variations regarding the levels of K^+ , Mg^{2+} , Ca^{2+} as well as the glucose. There is no "best" solution, most laboratories develop ACSF recipe by which could yield the most viable slices for electrophysiological recordings and produce the most stable synaptic events.

ACSF used in the present study contains the following components at final ion concentrations (in mM): 124 NaCl, 4.9 KCl, 1.2 KH_2PO4, 2.0 MgSO4, 2.0 $CaCl_2$, 24.6 $NaHCO_3$, and 10 D-glucose. All the chemicals are purchased from Applichem (Germany). Before using, ACSF was equilibrated with carbogen (95% O_2 and 5% CO_2), thereby adjusting its PH to 7.2-7.3.

2.2.2 Acute Hippocampal Slices Preparation

The unique structure and its role in memory identify the hippocampus as an ideal neural structure for the investigation of synaptic plasticity. In addition, anatomically, it could be isolated from the brain as a whole and sectioned transversely into slices like a loaf of bread, whereas the intrinsic circuitry could be kept preserved for many hours *in vitro* provided certain conditions are met (Figure 2. 2).

Hippocampal slice preparation is very useful for the investigation of synaptic function and plasticity mechanisms of hippocampus. Acute hippocampal slices were used for all electrophysiological recordings in the current study. All procedures of the slices preparation were carried out in compliance with the guidelines from the Animal Committee on Ethics in the Care and Use of Laboratory Animals of TU- Braunschweig.

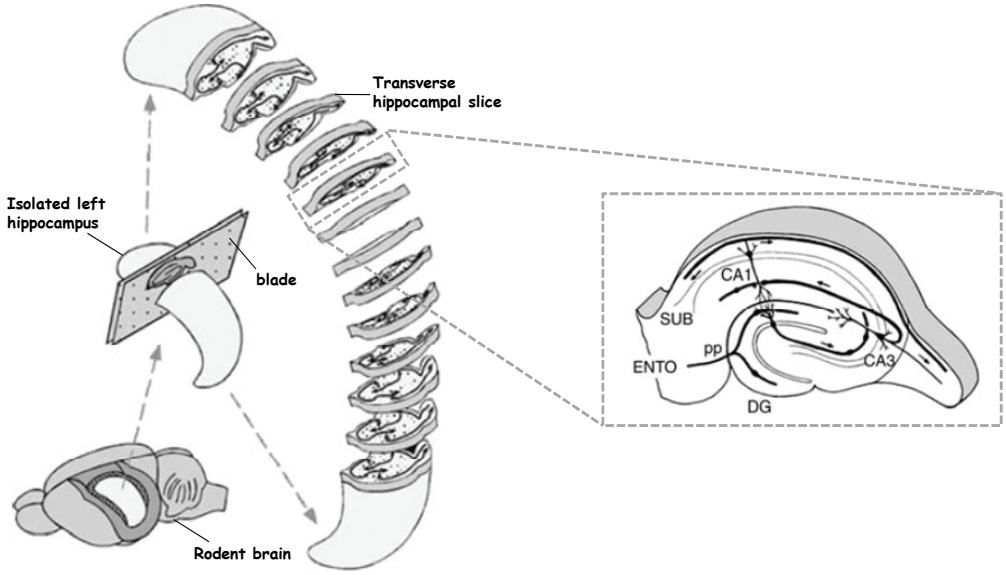


Figure 2. 2 | Hippocampal slices preparation.
Lower left: Schematic of rodent brain showing the localization of hippocampus on left side of brain (white). **Center**: Schematic of left hippocampus after isolation from brain and cutting of hippocampus into slices transverse to longitudinal axis. **Right**: Schematic of one transverse hippocampal slice showing the well-preserved intrinsic projections. (Source: Adapted from Akay, 2007).

Briefly, animals (either rats or mice) were anaesthetized in a chamber filled with 100% CO_2 and then decapitated immediately. Following decapitation, the scalp covering the skull was cut away and an incision was made on both sides. The whole skull plates covering the brain (occipital, parietal, and temporal skull plates) and dura near the temporal plate were then removed away, after which the whole brain was immersed into the cold (kept at 2-4 °C) and oxygenated ACSF in a petri dish covered with Whatman paper. The purpose of using 2-4 °C ACSF is to slow down the metabolism and minimize the deleterious events in the hippocampus which otherwise would cause cell death or tissue damage (Sajikumar et al., 2005a). For dissection of the hippocampus, the cerebellum and one quarter of the forebrain were firstly cut off. Then the two hemispheres were separated mid-sagitally by cutting along the interhemispheric fissure, after which the hippocampus was separated from the cortex and the underlying brainstem and midbrain. Once the hippocampus is free, gently position (at angle of 70°C to the long axis of the middle third of hippocampus) it on the stage of the manual tissue chopper (Stoelting co, USA) and sliced it rapidly into 400 µm thick transverse sections (Teyler, 1980; Sajikumar et al., 2005a) (Figure 2. 2). Hippocampal slices are then picked up gently by a wet paint brush and collected in a small beaker containing ice-cold and oxygenated ACSF.

2.2.3 Hippocampal Slices Incubation

Immediately after the preparation, hippocampal slices were transferred to an interface brain slice chamber (Scientific System Design, Canada) by a wide bored plastic pipette for incubation (Figure 2. 3). The slices were laid on a custom-made nylon net insert in the chamber where they are continuously perfused with carbogenoted ACSF (PH 7.2 to 7.3) at a flow rate of 0.79 ml/min. The temperature of the chamber was always maintained at 32 °C during all the stages of incubation and electrophysiological recordings by a heating element at the lower part of chamber controlled by the Proportional Temperature Controller (PTC) (Scientific System Design, Canada). It is worthwhile to mention that the interface chamber used in the current study allows to record very stable and long-term synaptic plasticity up to 12 h (see Sajikumar et al., 2014), which is hardly possible in the other commonly used submerged chamber. This is due to interface type chamber at the one hand, allows the oxygenated ACSF to reach the brain slice from the bottom, at the other hand, carbogen (through a bubbler located in the lower part of the chamber) also diffuse though a thin (50-200 μM) layer of ACSF that cover the surface of the slices, providing ideal conditions for maintaining the functionality of living neuronal tissue for many hours (Reid et al., 1988; Matthies et al., 1997). Whereas in submerged chamber, brains slices are supplied with oxygenation solely through the superfused ACSF flow with a higher flow rate of 1.5-3.0 ml/min, which limits the oxygen supply and therefore results in physiologically less ideal long-term plasticity recording conditions (Reid et al., 1988; Hajos and Mody, 2009).

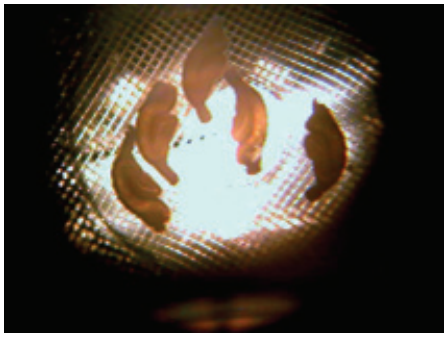


Figure 2. 3 | Incubation of hippocampal slices in an interface chamber.

The purpose of the incubation is to allow the hippocampal slices to reach metabolic stability so that more stable and reliable long-term recordings could be achieved (Ho et al., 2004; Sajikumar et al., 2005a). In the present study, hippocampal slices were preincubated in the interface chamber for 2-3 h before electrophysiological recordings – a rather long time period compared to most laboratories but is critical for long-term plasticity studies (Sajikumar et al., 2005a). The incubation time *in vitro* among different laboratories is of dramatically differences, from 30 min to 4 h. Nevertheless, it was reported that the incubation time of brain slices *in vitro* could have profoundly effects on the phosphorylation levels of synaptic proteins that involved in NMDA receptor-dependent

synaptic plasticity, including GluR1 subunit of AMPARs, CaMKII, ERK2 (Ho et al., 2004). In particular, LTD induced by LFS is surprisingly sensitive to the incubation time, the longer the incubation time *in vitro* are, the easier to get LTD (Ho et al., 2004).

2.3 Electrophysiology

2.3.1 Field Potential Recording

Excitatory postsynaptic potential (EPSP) is a transient postsynaptic membrane depolarization caused by a flow of positive charged ions into the postsynaptic cell due to the presynaptic release of excitatory neurotransmitter. Recoding extracellular EPSP of a single neuron is hardly possible as the signal from one single neuron activity is extremely small. However, in the brain area of hippocampus, the neurons are tightly packed together in the same orientation that they receive synaptic inputs in the same area. Thus, when stimulating a population of hippocampal neurons simultaneously, their individual EPSP can add together (spatial summation of EPSPs) to give a signal – the so called field EPSP (fEPSP) (Figure 2. 4), which could be recorded extracellularly by a field recording electrode.

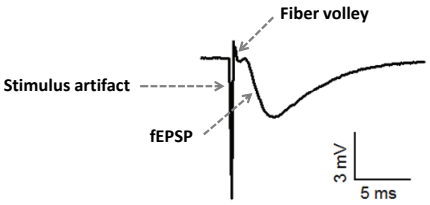


Figure 2. 4 | A trace of a field excitatory postsynaptic potential (fEPSP).

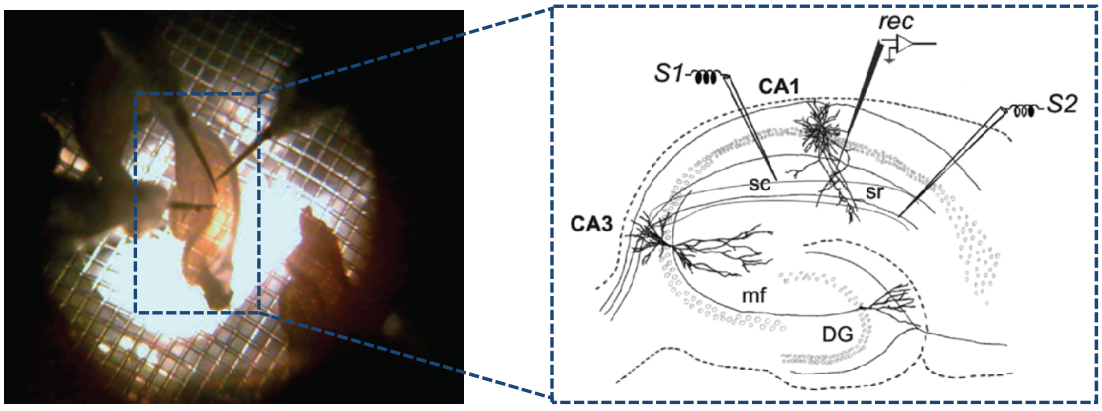


Figure 2. 5 | Recording fEPSP in a transversal hippocampal slice.
Left panel: Microscopic view of the two stimulating electrodes and one recording electrode located at the CA1 area of one hippocampal slice. **Right panel**: Schematic representation depicting the independent but convergent inputs onto pyramidal cells in the CA1 region of a hippocampal slice *in vitro*. The recording electrode (***rec***) placed in the stratum radiatum of CA1 records two independent fEPSPs elicited by the activation of two different populations of synapses (stimulation electrode 1, ***S1***; stimulation electrode 2, ***S2***) onto the same cells. DG, dentate gyrus; mf, mossy fiber; sc, schaffer collaterals; sr, stratum radiatum.

In the present study, fEPSPs were recorded from the CA1 apical dendritic layer (stratum radiatum) that received synaptic projections from CA3 via schaffer collateral commissural pathway (Figure 2. 5). In all the experiments, fEPSP recordings were carried out at 32 °C with constant perfusion of oxygenated ACSF at a flow rate of 0.79 ml/min. The neurons in the hippocampal slices could be visualized by a low magnification microscope which greatly favors the placing of the stimulating and recording electrodes. For evoking fEPSP response in a hippocampal slice, two monopolar lacquer-coated, stainless-steel electrodes (5 MΩ; AM-Systems, USA) were placed at an adequate distance within the stratum radiatum of the CA1 region to stimulate two independent synaptic inputs but converged to the same population of neuron (indicated in Figure 2. 5 as synaptic input (***S1***) and synaptic input (***S2***). For recoding the fEPSP, one recording electrode (Stainless-steel; 5 MΩ; AM-Systems, USA) was placed in the CA1 apical dendritic layer (indicated in Figure 2. 5 as ***rec***).

The initial slope of the fEPSPs (mV/ms) waveform is typically regarded as a good parameter for the investigation of synaptic strength in most laboratories. Although the absolute peak amplitude of fEPSP is also used as a measurement in some laboratories, it is easily subjected to contamination by the later stages of EPSP when the neuron fire action potentials. The initial slope of the fEPSP was measured in the current study.

2.3.2 Stimulation Protocols

The stimuli that delivered to hippocampal slices were generated from the isolated pulse stimulator (Model 2100, AM Systems) which triggered the application of the defined current in the desired programmed frequency.

Test Pulse Stimulation

Following the preincubation period, an input-output relationship (afferent stimulation intensity vs. fEPSP slope) was performed to get the maximal fEPSP slope. The test stimulation intensity strength for each synaptic input was determined to yield 40% of the maximal (fEPSP) slope and kept unchanged during the whole recording session.

Control stimulation at low rates could retain long-lasting network stability and increase viability of the slices from preparation so that the activity of kinase and other molecules drop to a resting state between stimulation, whereas control high rates stimulation results intrinsic metaplastic events which might influence the subsequently induced plasticity events (Schurr et al., 1986; Sajikumar et al., 2005a; Redondo and Morris, 2013). In the

present study, four 0.2 Hz biphasic, constant-current pulses (0.1 ms/polarity) were used per input pathway for baseline recordings and were delivered testing at 1, 3, 5, 11, 15, 21, 25, 30 min post-tetanus or 21, 25, 30 min post-LFS and thereafter every 15 or 5 min up to the end of the recording.

Late-LTP, Early-LTP

Either high-frequency stimulation (HFS) or theta-burst stimulation (TBS) can give rise to late-phase LTP (L-LTP) (Figure 2. 6). Although both of the two protocols could induce an L-LTP that is long-lasting, the underlying mechanisms of the two protocols induced L-LTP are different (Albensi et al., 2007). For instance, L-LTP triggered by repeated trains of HFS is translation, transcription and NMDAR dependent (Frey et al., 1988; Reymann and Frey, 2007). More importantly, HFS induced L-LTP is approachable to late associativity process of STC, and cross-capture (Frey and Morris, 1997; Sajikumar and Frey, 2004b; Redondo et al., 2010; Shires et al., 2012). Whereas TBS (5 Hz, 30 sec) triggered L-LTP is a local form (i.e., restricted locally to the dendritic compartment), and requires translation but not transcription. Of note, TBS induced L-LTP is not accessible to STC (Huang and Kandel, 2005; Sajikumar and Korte, 2011a). However, TBS pattern is believed to be more physiological, as it resembles the endogenous naturally firing pattern of hippocampal neurons during animal movement (Bland, 1986; Buzsaki, 2002). Moreover, TBS triggered LTP results more BDNF secretion compared with HFS-LTP (Gartner and Staiger, 2002).

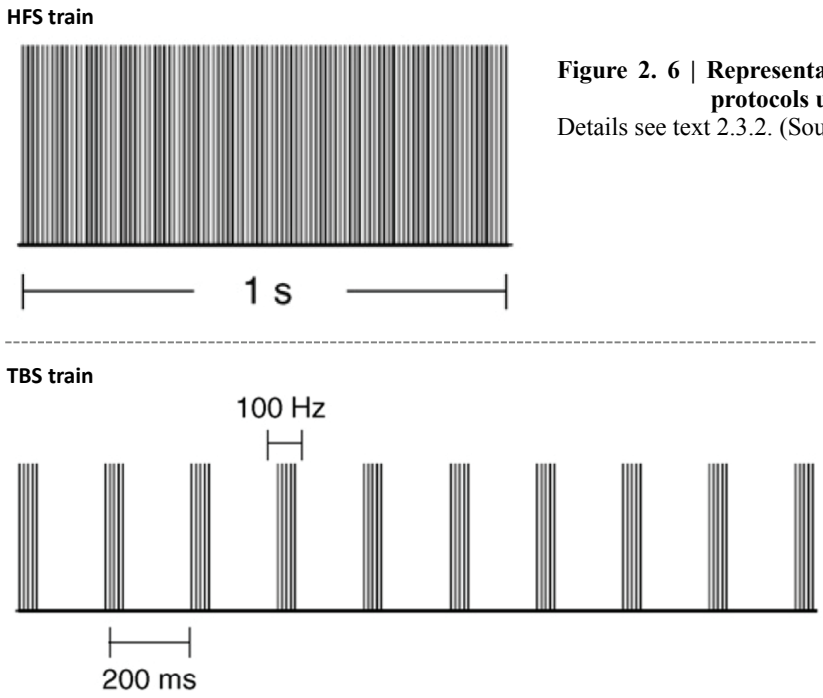


Figure 2. 6 | Representation of HFS and TBS protocols used to induce LTP.
Details see text 2.3.2. (Source: Raymond, 2007).

In the present study, Late-LTP (L-LTP) was induced by using 3 HFS trains of 100 pulses ("strong" tetanus [STET], 100 Hz; duration, 0.2 ms/polarity; intertrain interval, 10 min), which favors the investigation of late associativity process of STC. Early-LTP (E-LTP) was induced using a relatively weak stimulation pattern of 1 HFS train of 21 pulses ("weak" tetanization [WTET], 100 Hz; pulse duration, 0.2 ms/polarity).

Late-LTD

Low-frequency stimulation (LFS) is a widely used and an effective paradigm for LTD induction. In the present study, to induce late-LTD (L-LTD), a prolonged periods of strong low-frequency stimulation protocol (SLFS) consisting of 900 bursts (one burst consisted of 3 stimuli at a frequency of 20 Hz, interburst interval = 1 s, i.e. f = 1 Hz; stimulus pulse duration: 0.2 ms; thus a total number stimuli of 2700) was delivered within 15 min. The SLFS induced L-LTD has been proved to be dependent on protein synthesis and NMDAR activation (Mulkey and Malenka, 1992; Sajikumar and Frey, 2003, 2004b). Furthermore, it is accessible to STC and cross-capture (Sajikumar and Frey, 2004b).

Depotentiation (DP)

For inducing depotentiation (DP), low-frequency stimulation (LFS) was applied using 250 pulses at a frequency of 1 Hz, as the same used in previous studies (Sajikumar and Frey, 2004a; Sajikumar et al., 2009).

Synaptic Priming

Synaptic mGluR priming was elicited through the delivery of 2 TBS (2xTBS) in the presence of NMDA receptor antagonist AP-5 (50 μM), as reported previously (Raymond et al., 2000). AP was applied to prevent LTP induction. TBS consists of 10 bursts of stimulation at 100 Hz (5 biphasic pulses per burst), repeated at 5 Hz (theta bursts) with an interburst interval of 200 msec (Figure 2. 6), at the test pulse intensity. Two TBS consists of two trains of TBS, each train separated by 30 sec.

2.3.3 Rejection Criteria

Electrophysiological fEPSP recordings of hippocampal slices that showed large fiber volleys, maximal fEPSPs amplitude of less than 0.5 mV, unstable baseline, or substantial changes in the fiber volley during recording were rejected.

2.3.4 Data Acquisition and Analysis

The recorded signals (i.e., extracellularly fEPSPs) were firstly amplified by a differential amplifier (Model 1700, AM Systems) and then digitized by a CED 1401 analog-to-digital (A/D) converter (Cambridge Electronic Design). Following digitization, the signals were transferred to a computer for on-line and off-line analysis by custom-made software. For the measurement of synaptic strength, the initial slopes of the evoked fEPSPs per recording time point were normalized to baseline and calculated as (fEPSP slope per recording time point /mean EPSP slope baseline) × 100.

All graphs were generated using GraphPad Prism 5, data were represented as mean ± S.E.M (standard error of the mean).

2.4 Pharmacology

The drugs used in the current study were dissolved in either DMSO (dimethyl sulfoxide) or distilled water as concentrated stock solutions and stored at -20 °C or 4 °C as required. Immediately before each bath application, the drugs are prepared from stock solution and dissolved in ACSF. The final concentration of DMSO was always 0.1%, which has been proved to have no effect on basal synaptic transmission (Navakkode et al., 2004).

RyR agonist

To investigate the priming effect by pharmacological RyR activation, RyR agonist ryanodine (RYA, 10 μM, dissolved in DMSO; Tocris) was used as previously reported (Sajikumar et al., 2009). Ryanodine is a compound which has extremely high affinity to the open-form of RyRs. It can modulate RyRs at a dose-dependent dual manner. Specifically, low dose of RYA (from nanomolar to 10 μM) lock the RyRs irreversibly into a subconductance (open state), whereas high concentrations (~100 μM) of it fully block the channel-opening (Meissner, 1986; McPherson et al., 1991). To check whether RYA has any long-term nonspecific effect of on the stability of the synaptic potentials, RYA was applied for 30 min after a 30 min baseline of synaptic input S1 and S2. Both S1 and S2 showed stable potentials for 6 h (Figure 2. 7), which excludes the possibility that RYA has non-specific effects on synaptic efficiency.

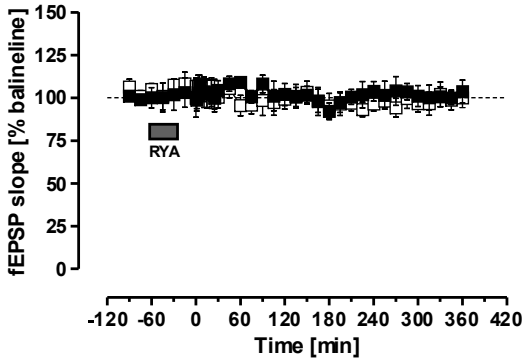


Figure 2. 7 | RYA has no long-term non-specific effects on synaptic efficiency.
Control baseline response from two independent synaptic inputs S1 and S2 (black and white squares) shows stable potentials up 6 h. RYA (10 μM) was bath-applied 30 min after a stable baseline for the next 30 min (n = 5). Both inputs S1 and S2 show stable baseline potentials during the entire recording period of 6 h.

Protein synthesis inhibitors

Anisomycin (ANI; Tocris) was dissolved in DMSO as stock solution and used at a concentration of 25 μM. It was reported earlier that anisomycin at this concentration blocks at least 85% of [3H] leucine incorporation (as a measure for protein synthesis) into hippocampal slices (Frey et al., 1991). It interferes with protein synthesis by inhibiting peptidyl transferase or the 80S ribosome system. It is well established that anisomycin can block LTP or LTD maintenance both *in vivo* and *in vitro* (Krug et al., 1984; Frey et al., 1988; Sajikumar and Frey, 2003; Kelleher et al., 2004). In addition, its injection into hippocampus inhibits the consolidation of long-term spatial memory (Meiri and Rosenblum, 1998).

However, it should be noted that in addition to its protein synthesis inhibition effect, anisomycin is a potent activator of the p38 MAPK and JNKs (c-Jun N-terminal kinases) pathway (Hazzalin et al., 1998; Croons et al., 2009). Therefore, to ensure specificity, a structurally different irreversible protein synthesis inhibitor, emetine (EME, 20 μM, dissolved in DMSO; Sigma), was also used in some experiments. Emetine inhibits protein synthesis by binding to the 40S ribosomal subunit and inhibiting translocation (Grollman, 1966; Jimenez et al., 1977).

PKMζ inhibitor

ZIP (zeta inhibitory peptide) is a widely used PKMζ inhibitor. It is a 13-amino-acid sequence thought to mimic the natural substrate that turns PKMζ off by providing the auto inhibition of the missing PKCζ regulatory domain (Figure 2. 8) (Pastalkova et al., 2006; Yao et al., 2013). There are strong evidences showing that pharmacological inhibition of PKMζ by ZIP could reverse LTP maintenance (Ling et al., 2002; Serrano et al., 2005) and erase consolidation of long-term memory (Shema et al., 2007; Serrano et al., 2008;

Sacktor, 2011). However, two recent studies questioned the specificity of ZIP on PKMζ as they provided evidence that ZIP could reverse the intact LTP as well as hippocampal dependent memory tasks in constitutive PKCζ/PKMζ KO mice (Lee et al., 2013; Volk et al., 2013). Nevertheless, compensatory increase of the other atypical PKC isoform, PKCλ/ι, was found in these constitutive PKCζ/PKMζ KO mice (Tsokas. et al., Society for Neuroscience Annual Meeting, New Orleans, LA, 2012; Tsokas. et al., Proceedings of the 9th FENS Forum of Neuroscience, Milan, Italy, 2014). PKCλ/ι is a molecule which also involves in LTP expression and notably can be reversed by ZIP at concentration of more than 2.0 µM (Ren et al., 2013). Lee et al., (2013) used ZIP at a concentration of 4-5 µM which very likely targets PKCλ/ι due to the compensatory increase in constitutive PKCζ/PKMζ KO mice. Future study will be needed to examine the extent to which ZIP inhibits PKMζ and PKCλ/ι. (For more details, see section 4.6).

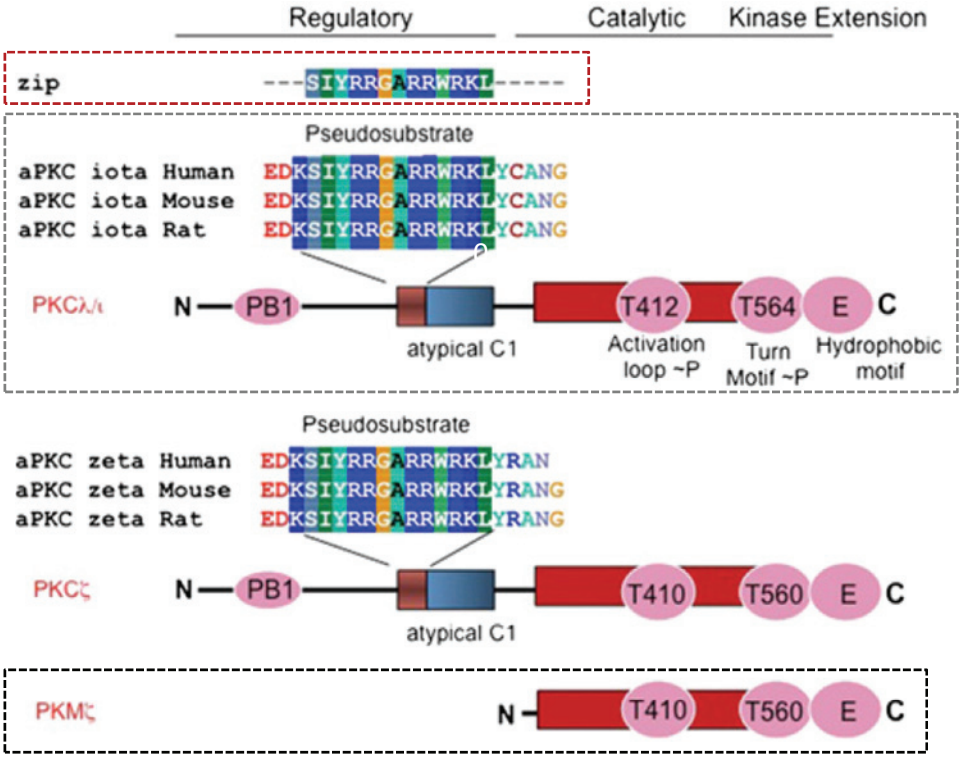


Figure 2. 8 | Schematic of the pseudosubstrate inhibitor ZIP and the protein structure for atypical PKC isoforms.
Details see text. (Source: Price and Ghosh, 2013).

In the present study, myristoylated pseudosubstrate peptide myr-ZIP (myr-SIYRRGARRWRKL-OH, dissolved in distilled water; AnaSpec) was used at a dose of 1 µM, as the same used in previous studies (Sajikumar et al., 2007; Sajikumar and Korte, 2011b, a). Its corresponding scrambled control peptide scambled-ZIP (scr-ZIP)

(myr-RLYRKRIWRSAGR-OH, dissolved in distilled water; synthesized from Peptide 2.0) was also used at a dose of 1 μM, as reported previously (Sajikumar and Korte, 2011a).

CaMKII inhibitor

The selective, cell-permeable inhibitor of CaMKII, KN-62 (1-[NO-bis-1, 5-isoquinolinesulfonyl]-N-methyl-L-tyrosyl-4-phenylpiperazine; Calbiochem) was dissolved in DMSO as stock solution (1 mM) and diluted in ACSF to reach the concentrations of 5 μM immediately before bath application, as the same used in previous studies (Sajikumar et al., 2007; Sajikumar et al., 2009).

NMDAR antagonist

NMDAR antagonist D-2-Amino-phosphonopentanoic acid (AP-5) (Tocris) was prepared in DMSO as stock solution and used at a concentration of 50 μM as previously reported (Korte et al., 1995; Navakkode et al., 2005, 2007).

Group 1 mGluR antagonist

AIDA, (R, S)-1-aminoindan-1, 5, dicarboxylic acid (Tocris), was prepared in DMSO as stock solution and used at a concentration of 500 μM to block group 1 mGluR as previously reported (Oliet et al., 1997; Raymond et al., 2000).

2.5 Western Blot

Sample collection

All the samples used for western blot analysis of PKMζ were collected from CA1 regions of hippocampal slices. The hippocampal slices were prepared in the same way as that of for the electrophysiological recordings. For probing whether RyR priming leads to PKMζ synthesis in physiological conditions, The following 5 groups of slices were subject to western blot analysis (Figure 3. 7E): 1) control slices which were incubated in the interface chamber for 3 h; 2) E-LTP group; 3) RyR-primed E-LTP group; 4) myr-ZIP together with RyR- primed E-LTP group; and 5) Anisomycin together with RYR-primed E-LTP group. In each group, 18 slices from Wistar rats were used. For checking the PKMζ expression level in the hippocampal-CA1 in APP/PS1 mice, two groups of acute hippocampal slices CA1 region were collected without preincubation and stimulation (see also Figure 3. 15A): 1) WT group; 2) APP/PS1 group. To study whether RyR priming leads to protein synthesis of

PKMζ in APP/PS1 mice, the following 6 groups of slices were collected (see also Figure 3. 15B): 1) WT-L-LTP; 2) APP/PS1-L-LTP; 3) RyR primed APP/PS1-L-LTP; 4) anisomycin together with RyR primed APP/PS1-L-LTP; 5) myr-ZIP together with RyR primed APP/PS1-L-LTP; 6) scr-ZIP together with RyR primed APP/PS1-L-LTP. In each group, 12 to 16 slices from APP/PS1 or WT mice were used for the studies. CA1 region of slices of the above groups were quickly dissected on ice 1 h after the induction of L-LTP and used for further analyses. After collection, the samples were firstly put in liquid N_2 for deep frozen and then kept in -80 °C for further analysis.

Western blot

For the western blot analysis, pooled hippocampal CA1 regions of each group were collected in STKM buffer (250 mM saccharose, 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 1 μM leupeptin, 1 μM pepstatin A, 0.4 mM 4--(2-aminoethyl)-benzolsulfonylfluoride (AEBSF), 1 μM aprotinin) and lysated by 3 freeze/thaw cycles. After centrifugation for 10 min at 13 000 × g, the protein concentration of the supernatant was determined using Bradford assay. Four micrograms of total protein were subjected to SDS-PAGE and subsequent immunoblotting with anti-bodies against PKMζ (38-1400; Invitrogen), tubulin (DM1A; Sigma-Aldrich) or GAPDH (Acris), respectively. The amount of PKMζ was quantified by densitometric measurement of western blots using EasyWin (Herolab, Germany). The densitrometric values of each blot were normalized to the amounts of tubulin or GAPDH which served as a loading control and were calculated in relation to the control group. The values of each data points were represented as mean of at least 3 independent experiments, using independent samples.

2.6 Statistical Analysis

The calculated values (percentage changes from the baseline mean) of the slope of the fEPSP (mV/ms) per recording time point were analyzed using the Wilcoxon signed-rank test (Wilcoxon test) when compared within the group (i.e., baseline and post stimulus), or the Mann-Whitney *U* test (*U* test) when data were compared between the groups (i.e., the two independent pathways S1 and S2). $P < 0.05$ was regarded as being statistically significant different. For comparing the difference of the western blot analysis of PKMζ results, t-test or one-way ANOVA with Dunnett's post hoc tests at the $P < 0.05$ significance level was used in Prism 5.

3. RESULTS

In the present study, the effects of metaplasticity on hippocampal synaptic plasticity were investigated. The first section (3.1 Metaplasticity Prolongs Late Associativity) of the work deals with metaplasticity by RyR or synaptic mGluR activation on late associativity process of STC (data are already published Li et al., 2014), and the second part is about metaplasticity by RyR activation in preventing the synaptic impairments in AD (3.2 Metaplasticity Prevents Synaptic Plasticity Deficits in an Alzheimer's Disease Mouse Model). All the experiments were performed in the acute hippocampal slices of rats (result 3.1) or mice (result 3.2).

3.1 Metaplasticity Prolongs Late Associativity

Revealed by a "strong-before-weak" experimental paradigm, so far STC has been observed for a limited time window of 60 min. This is due to the decay nature of the synaptic tag mediated by CaMKII. Metaplasticity by RyR activation has been demonstrated to lower the threshold of STC by creating new synaptic tag. The present study provide compelling evidences that priming RyR or mGluR activation has substantial effects on the synaptic tag setting process.

3.1.1 RyR or mGluR Priming Facilitates E-LTP

It was reported earlier that metaplasticity of synapses in hippocampal CA1 by pharmacological activation of RyR via its agonist, e.g., ryanodine (RYA; 10 μM) or caffeine (10 mM), facilitates ("primes") both the induction and persistence of subsequent STP (Sajikumar et al., 2009). In the present study, the priming effect of RYA on the classical early-LTP (E-LTP) was investigated. In a control experiment, after recording a stable baseline in both synaptic inputs S1 and S2 for 1 h, weak tetanization (WTET) was applied to S1 for E-LTP induction. As shown in Figure 3. 1A, WTET of S1 triggers a transient E-LTP lasting nearly 210 min (*U* test, $P = 0.02$; Wilcoxon test, $P = 0.01$), while in the control synaptic input S2, the potentials were stable during the entire recording time period. In the next series of experiments, the effect of RYA (10 μM) priming on E-LTP was examined. After recording a stable baseline in both S1 and S2 for 30 min, the hippocampal slices were primed by bath-application of RYA (10 μM) for 30 min and then WTET was applied to S1 thirty min after the drug washout (thus a total of 90 min baseline was

recorded). Consistent with previous reports (Mellentin et al., 2007; Sajikumar et al., 2009), here both the induction and persistence of E-LTP were significantly facilitated by RyR priming without having any effect on the potentials of synaptic input S2 (Figure 3. 1B). Statistically significant potentiation was observed in synaptic input S1 up to 285 min by *U* test ($P = 0.02$) and 300 min by Wilcoxon test ($P = 0.04$).

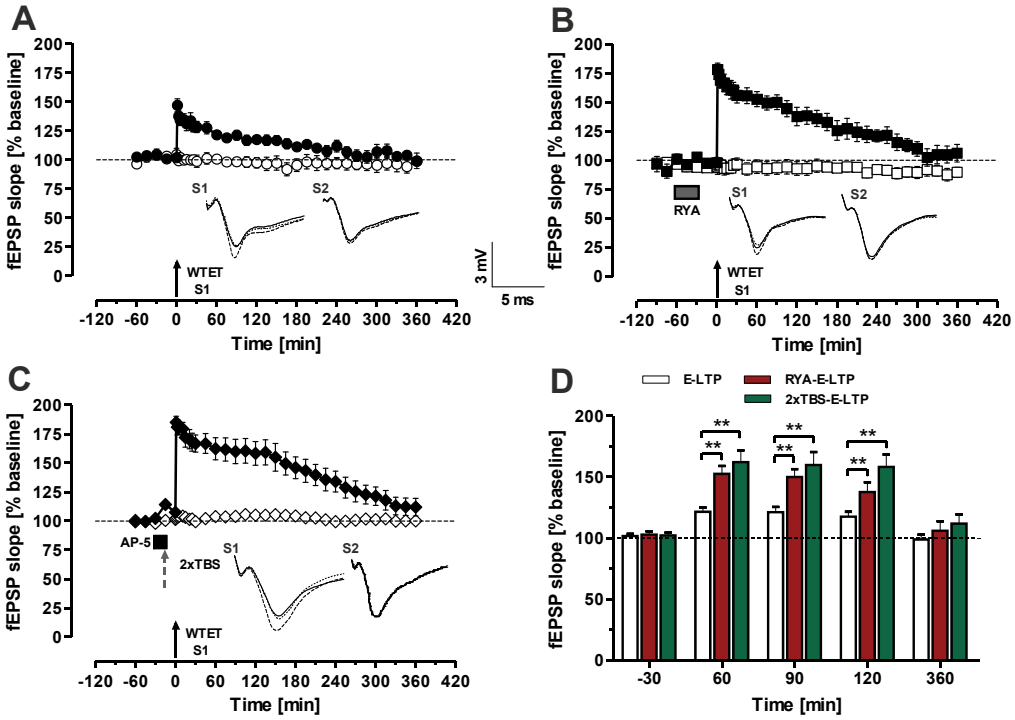


Figure 3. 1 | Metaplasticity of E-LTP.
(A) After recording a stable baseline of 60 min, WTET was applied to synaptic input S1 (black circles) which resulted in a transient LTP lasting 210 min. Baseline potentials recorded from S2 (white circles) showed stable potentials during the entire recording period (n = 7). **(B)** Application of ryanodine (RYA; 10 µM) for 30 min, and then washout for 30 min prior to E-LTP induction increased the persistence of E-LTP to 300 min in S1 (black squares) without affecting the potentials in S2 (white squares) (n = 7). **(C)** Bath application of AP-5 (50 µM) for 10 min and priming stimulation by 2xTBS (gray broken arrow) in the presence of AP-5 followed by the application of WTET 20 min after AP-5 washout resulted in a significantly enhanced E-LTP lasting 300 min (black diamonds, n = 7). **(D)** Summary bar graph shows differences in the percentage of S1 potentiation at -30, 60, 90, 120, and 360 min after the induction of E-LTP, RYA and 2xTBS primed E-LTP that are presented in (A–C). The asterisk in 60, 90, and 120 min represents statistically significant potentiation (**P < 0.01 by *U* test) with the compared group. Single arrow represents weak tetanization (WTET) applied for inducing E-LTP. Insets in each graph represent typical fEPSP traces recorded from synaptic inputs S1 and S2 thirty min before (dotted line), 30 min after (broken line), and 6 h after (full line) the induction of corresponding plasticity, respectively. All data are plotted as mean ± SEM. Error bars indicate SEM. Calibration bar for all analog sweeps: 3 mV/5 ms.

It has been reported previously that LTP can also be primed by synaptically released glutamate through mGluR-mediated mechanisms (Raymond et al., 2000). To test this in the current experimental condition and make the findings more physiologically relevant, the

slices were primed by synaptically activating mGluRs through the delivery of 2xTBS but in the presence of the NMDA receptor antagonist AP-5 (50 μM) to prevent LTP induction and permit selective activation of the mGluRs. After a stable baseline of 30 min, AP-5 was bath-applied for 10 min and then washed out for 20 min before the delivery of WTET for inducing E-LTP. Intriguingly, similar to the effect of RYA priming on E-LTP, synaptic priming significantly facilitated the subsequent E-LTP lasting 300 min (Figure 3. 1C, *U* test, $P = 0.01$; Wilcoxon test, $P = 0.02$). Primed E-LTP elicited by either pharmacological activation of RyR or synaptic mGluR activation showed significantly enhanced potentiation up to 105 min compared with control E-LTP (Figure 3. 1D) [*U* test, 60 min ($P = 0.003$), 90 min ($P = 0.004$), 105 min ($P = 0.02$)]. Thus, RyR activation or 2xTBS priming before the induction of E-LTP facilitates E-LTP and results in an intermediate form of LTP, lasting nearly 5 h.

Of note, large elevations of intracellular calcium either via very high-frequency stimulation (HFS) of afferent fibers or via intracellular Ca^{2+} store like RyR, are known to induce NMDAR-independent LTP (Wang et al., 1996; Raymond, 2008). However, the RYA-primed E-LTP in the current study is NMDAR dependent (Figure 3. 2), an important prerequisite for STC (O'Carroll and Morris, 2004).

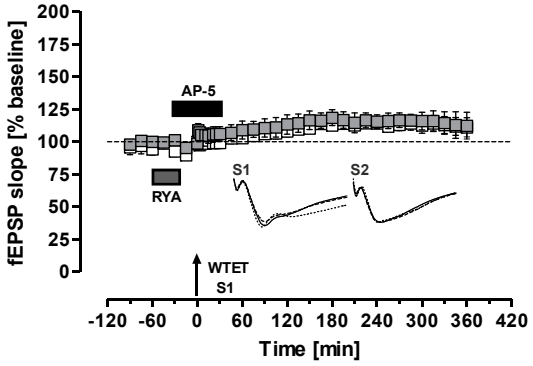


Figure 3. 2 | RYA-primed E-LTP is NMDAR dependent.
RYA priming followed by NMDAR blockade by AP-5 (50 μM) for 1 h and induction of E-LTP by WTET 30 min after AP-5 application. Here the induction and persistence of primed E-LTP was completely blocked (gray squares) without affecting the control potentials in S2 (white squares, n = 7). Symbols and traces as in Figure 3. 1

3.1.2 Associative Properties of Primed E-LTP

The "weak-before-strong" STC paradigm, i.e., weak tetanus (WTET) of one synaptic input prior to strong tetanus (STET) of an independent second synaptic input, reveals the synaptic tag duration in STC (Frey and Morris, 1998a; Sajikumar and Frey, 2004b). It has been demonstrated that the effective time course for the tag-PRP interaction is approximately 1 h under *in vitro* conditions at 32 °C (Frey and Morris, 1998a; Redondo and Morris, 2011). Since RyR priming of E-LTP results in an intermediate form of LTP, it

is of great interest to test how long the synaptic tag can persist in RYA or 2xTBS-primed E-LTP. To address this question, a series of control experiments for testing whether STC could occur between the WTET and STET episodes at the longer interval of 240 min was performed. Consistent with earlier findings, the expression of E-LTP by WTET of S1 could not be transformed into L-LTP if the subsequent induction of L-LTP occurred 240 min later in S2 (Figure 3. 3A). Statistically significant potentiation was observed only up to 180 min after the induction of E-LTP (Wilcoxon test, $P = 0.02$). To check whether RyR or 2xTBS-primed E-LTP can take part in the processes of STC with an interval of 240 min, the slices were primed by a 30 min-RYA bath application and then followed by 30 min drug washout, after which E-LTP in S1 was induced by WTET. As shown in Figure 3. 3B, primed E-LTP declined to baseline within 240 min, statistically significant potentiation was observed up to 210 min (Wilcoxon test, $P = 0.02$). Subsequently (i.e., 240 min after the induction of E-LTP in S1), L-LTP was induced in S2 by STET. Surprisingly, the decayed primed E-LTP in S1 slowly recovered to its initial potentiation level from 275 min onwards (Wilcoxon test, $P = 0.04$) until 480 min (Wilcoxon test, $P = 0.02$), thus expressing STC (Figure 3. 3B). To test whether 2xTBS primed E-LTP also takes part in STC at the late phase of 240 min as that of RYA priming, a similar experimental paradigm was used with the exception that instead of a 30 min bath application of RYA, 2xTBS was applied in the presence of AP-5 (50 μM). Likewise, 2xTBS primed E-LTP in S1 was transformed into L-LTP after the induction of L-LTP in S2 at 240 min, expressing STC (Figure 3. 3C). Potentials in S1 showed statistically significant potentiation from the time point of E-LTP induction until 480 min (Wilcoxon test, $P = 0.02$). L-LTP in S2 in Figure 3. 3A–C showed statistically significant potentiation from time point of tetanization until 480 min in (Wilcoxon test $P = 0.01$). Potentiation 8 h after the induction of either RYA primed E-LTP or 2xTBS primed E-LTP showed statistically significant potentiation in comparison to non-primed control E-LTP (Figure 3. 3E, *U* test, $P < 0.01$).

To confirm whether synaptic stimulation by 2xTBS specifically activates mGluRs in the current experimental condition similar to that of an earlier report (Raymond et al. 2000), priming stimulation in the presence of group 1 mGluR antagonist AIDA (500 μM) was applied during 2xTBS priming. In consistent with the result of Raymond et al., (2000), AIDA abolished the effects of 2xTBS priming as both the enhancement of E-LTP and its STC were prevented (Figure 3. 3D and E). Statistically significant potentiation was observed only up to 225 min in S1 (Wilcoxon test, $P = 0.04$). Potentials in S2 showed significant increases from the time of tetanization until 480 min (Wilcoxon test, $P = 0.01$).

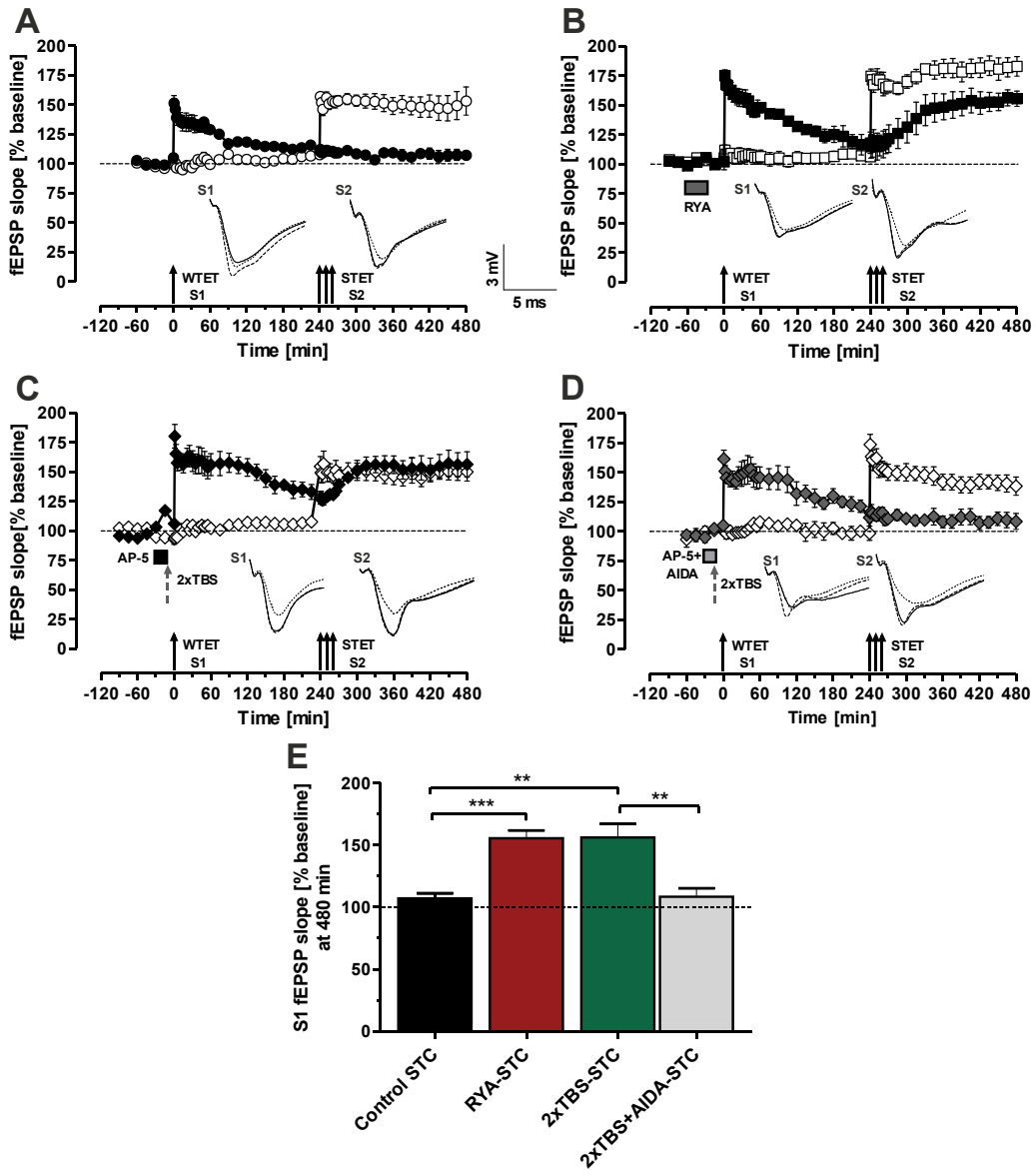


Figure 3. 3 | Primed E-LTP and STC.
(A) After recording a stable baseline of 60 min, WTET for E-LTP induction was applied to S1 (black circles), 4 h after which STET for L-LTP induction was delivered to S2. Here E-LTP in S1 failed to be converted to L-LTP showing no STC (n = 7). **(B)** Priming of E-LTP by bath application of RYA (10 μM) for 30 min, followed by a washout for 30 min prior to E-LTP (black squares) induction and subsequent induction of L-LTP (white squares) in S2 at 240 min. Primed E-LTP was transformed into L-LTP expressing STC (n = 7). **(C)** Experimental design as of (A) but priming was carried with synaptic activation by 2xTBS in the presence of AP-5. Similar to (A), the E-LTP in S1 (black diamonds) was transformed into L-LTP (n = 7). **(D)** Priming was carried with synaptic activation by 2xTBS in presence of AP-5 and mGluR type-1 antagonist AIDA (500 μM) (n = 5). No synaptic tagging and capture was observed. **(E)** Summary bar graph showing differences in the percentage of S1 potentiation at 8 h between the four different conditions presented in (A–D). The potentiation of S1 at 8 h in either RYA primed (B) or 2xTBS primed (C) conditions is significantly higher than the control unprimed (A) and, 2xTBS primed but AIDA inhibited conditions (D) (**$P < 0.01$; ***$P < 0.001$ by *U* test). Symbols and traces as in Figure 3. 1. In addition triplets of arrows represent strong tetanization (STET) for inducing L-LTP and full line in the insets represents traces recorded at 8 h.

In a critical next series of experiments, the time interval of L-LTP induction in S2 was increased from 240 to 300 min or 360 min. At the 300 min interval (Figure 3. 4A and C), the potentials in S1 decayed to baseline within 240 min but regained to original potentiated levels from 325 min onwards (significant potentiation from 325 to 540 min; Wilcoxon test, $P = 0.01$), expressing STC. However, at an interval of 360 min (Figure 3. 4B and C) the primed-LTP failed to show STC. Statistically significant potentiation up to 270 min was observed in primed E-LTP (*U* test, $P = 0.03$; Wilcoxon test, $P = 0.04$). L-LTP in S2 showed significant potentiation compared with their pre-potentiation values at all time points in these two experiments (Figure 3. 4A and B, Wilcoxon test, $P = 0.02$). Taken together, these experiments reveal a very interesting aspect of STC: RYR or 2xTBS priming prolongs the duration of the synaptic tags from 1 to 5 h.

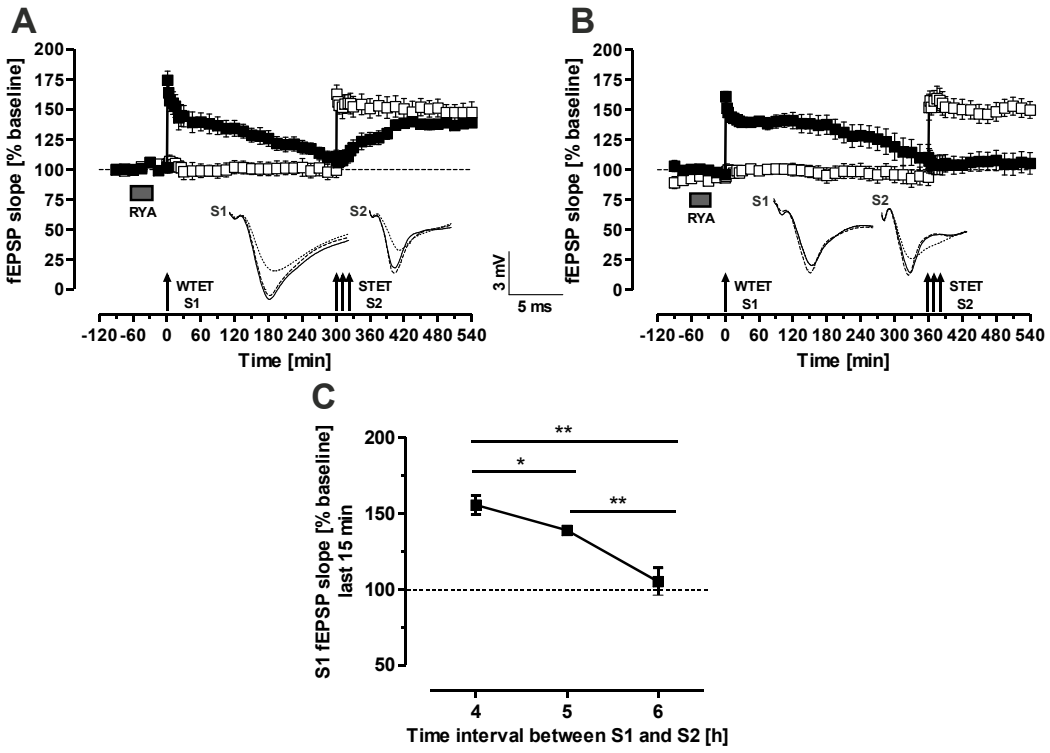


Figure 3. 4 | Decay time course of primed synaptic tag.
(A, B) Priming of E-LTP by bath application of RYA (10 µM) for 30 min, followed by a washout for 30 min prior to E-LTP (black squares) induction and subsequent induction of L-LTP in S2 (white squares) at 300 min (A) or 360 min (B). Here, primed E-LTP was transformed into L-LTP expressing STC in (A) (n = 7), but no STC was observed in (B) (n =7). **(C)** Summary graph showing differences in the percentage of S1 potentiation from the last 15 min recordings presented in (A, B) and Figure 3. 3B (*$P < 0.05$; **$P < 0.01$ by *U* test). The magnitude of S1 potentiation at the last 15 min decreases with the increased "weak before strong" interval. Symbols and traces as in Figure 3. 3. In addition full line in the insets represents traces recorded at 9 h.

3.1.3 Mechanism of RyR or mGluR Priming

It was well established that prior pharmacological or synaptic activation of group 1 mGluRs triggers *de novo* protein synthesis, with the newly synthesized proteins being kept in reserve and act as PRPs for the utilization of the subsequent induced synaptic events such as LTP induction (Raymond et al., 2000; Sajikumar and Korte, 2011a). The current findings show that the protein synthesis inhibitors anisomycin (ANI; 25 µM) or emetine (EME; 20 µM) during RYA priming abolished this priming effect, supporting the role of newly synthesized proteins (Figure 3. 5A–C). Statistically significant potentiation was observed in S1 only up to 180 min in Figure 3. 5A and up to 120 min in Figure 3. 5B (Wilcoxon test, $P = 0.04$). In both cases, L-LTP in S2 showed statistically significant potentiation from time point of tetanization until 480 min (Wilcoxon test $P = 0.02$).

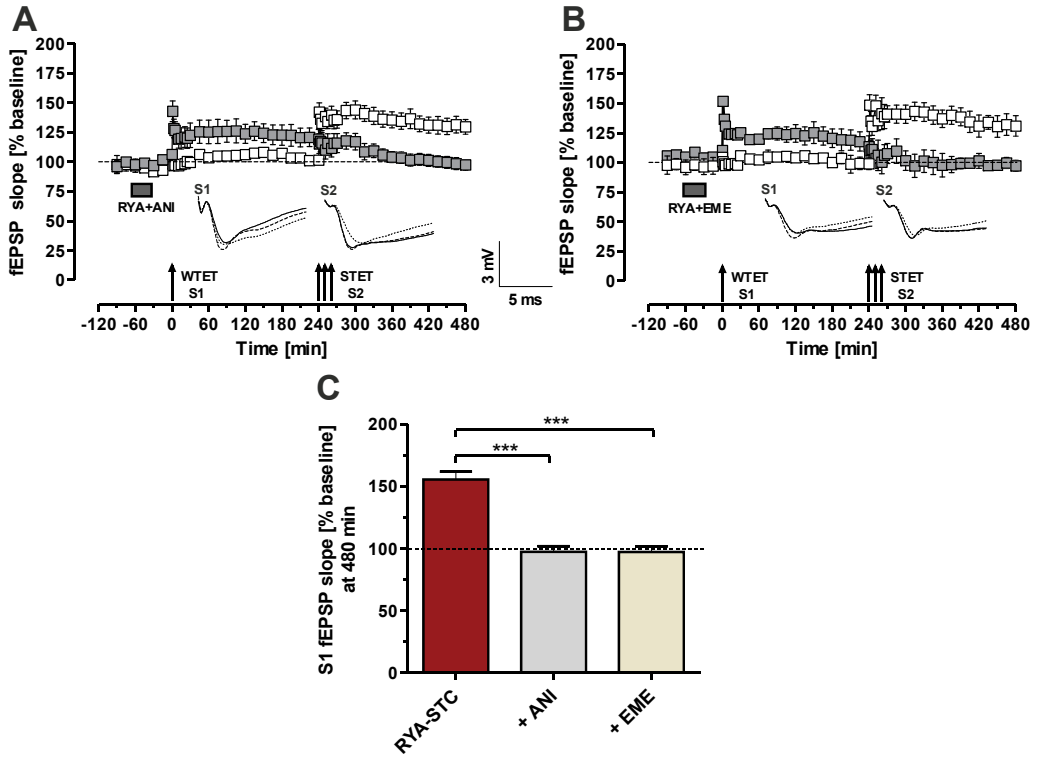


Figure 3. 5 | RyR priming of STC requires protein synthesis.
(A) Priming of E-LTP by bath application of RYA (10 µM) together with protein synthesis inhibitor anisomycin (ANI; 25 µM) for 30 min, followed by a washout for 30 min prior to E-LTP (gray squares) induction and subsequent induction of L-LTP (white squares) in S2 at 240 min. Primed E-LTP was not transformed to L-LTP (n = 5). Here no tagging and capture interactions observed. **(B)** Experimental design same as in (A) but RYA was co-applied with emetine (EME; 20 µM). Similar to (A), no STC was observed (n = 5). **(C)** Summary of S1 potentiation at 8 h shows protein synthesis inhibitors during RYA priming (A, B) prevents the facilitation of STC (***$P < 0.001$ by *U* test). Symbols and traces as in Figure 3. 3.

PKMζ has been proved to be one of the PRPs synthesized by pharmacological activation of group 1 mGluRs via its agonist DHPG (Sajikumar and Korte, 2011a). Since RyRs activation acts downstream of group 1 mGluR mediated priming of LTP (Mellentin et al., 2007), it is reasonable to hypothesize that prior RyR activation or synaptic priming of mGluR facilitates E-LTP also through *de novo* protein synthesis of PKMζ. To test this, a cell permeable and selective PKMζ inhibitor myr-ZIP (1 μM) was initially bath applied for 30 min and then coapplied with either RYA for 30 min (Figure 3. 6A) or AP-5 for 10 min (Figure 3. 6B). The peptide myr-ZIP was bath-applied before RYA application because this inhibitory peptide requires some time to incorporate into the cell to sufficiently block the signaling of PKMζ (Sajikumar et al., 2005b; Sajikumar and Korte, 2011a). In good agreement with the hypothesis, the persistence of RYA or 2xTBS-primed E-LTP in the presence of ZIP was similar to the control E-LTP in non-primed slices, without affecting the baseline in S2 (Figure 3. 6A and B). Statistically significant potentiation was observed in S1 up to 210 min in Figure 3. 6A (*U* test, $P = 0.02$; Wilcoxon test, $P = 0.01$) or up to 180 min in Figure 3. 6B (Wilcoxon test, $P = 0.03$) or 135 min (*U* test, $P = 0.04$). A comparison of primed E-LTP with primed but myr-ZIP inhibited E-LTP is presented in Figure 3. 6C.

Since prior RyR activation or synaptic activation of mGluR facilitates the subsequent E-LTP by protein synthesis of PKMζ, it is of interest to test furthermore whether the two priming induced synthesis of PKMζ regulates STC. As shown in Figure 3. 6D and E, co-application of myr-ZIP (1 μM) either with RYA or with 2xTBS abolished the primed STC, indicating PKMζ synthesized during priming is critical for primed STC. In Figure 3. 6D, S1 showed significant potentiation up to 270 min (Wilcoxon test, $P = 0.01$) or up to 225 min (*U* test, $P = 0.01$). In Figure 3. 6E, S1 showed significant potentiation only up to 195 min (Wilcoxon test, $P = 0.01$; *U* test, $P = 0.04$). In both cases, potentials in S2 showed significant increases from the time of tetanization until 480 min later (Wilcoxon test, $P = 0.01$). A comparison of S1 potentiation at 480 min in primed STC with primed but myr-ZIP inhibited STC is presented in Figure 3. 6F. Collectively, these results show that RyR or 2xTBS priming exerts its effect on E-LTP and its STC by *de novo* protein synthesis of PKMζ.

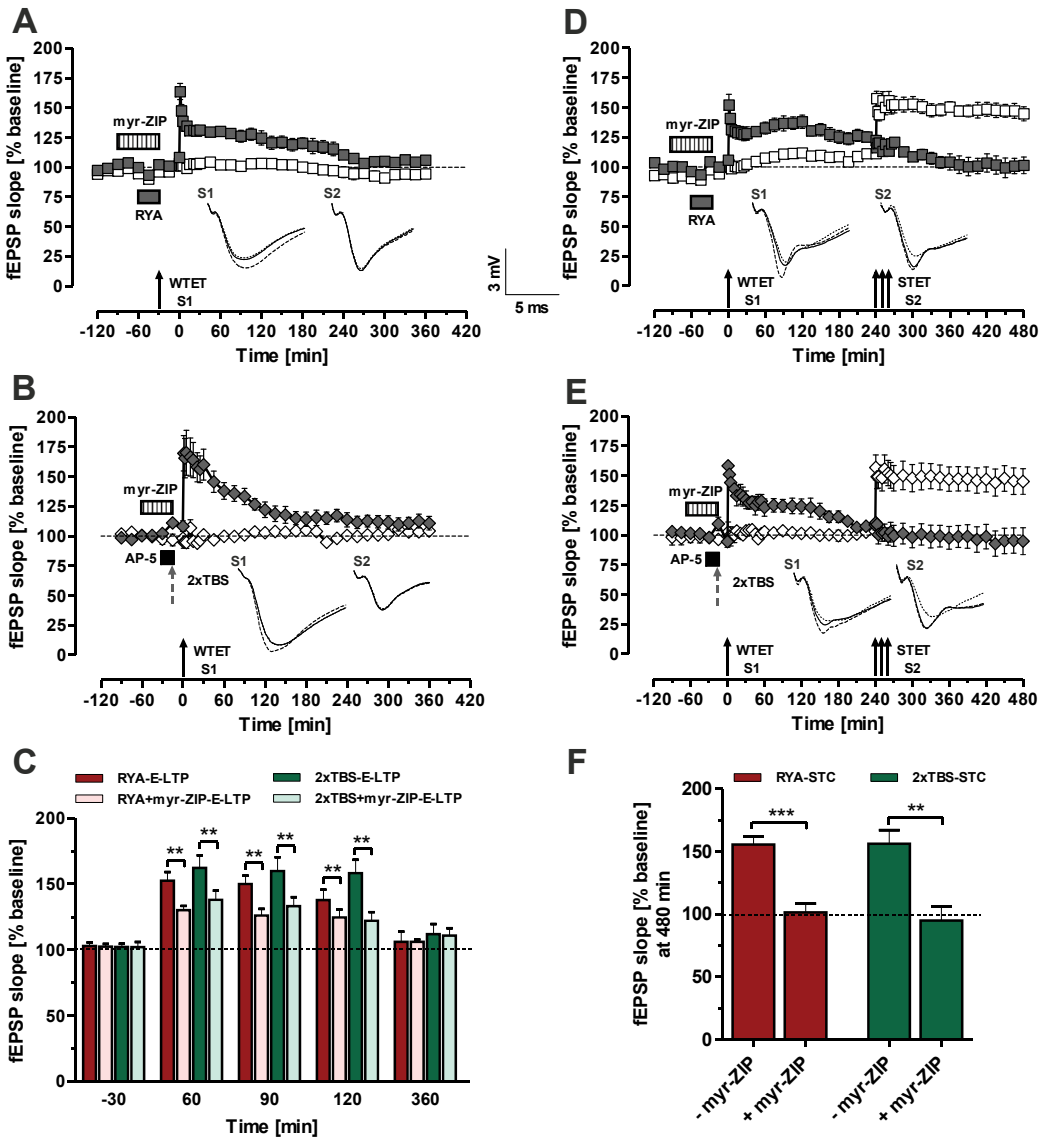


Figure 3. 6 | Mechanisms of RyR or mGluR priming.
(A) Application of PKMζ inhibitor myr-ZIP (1 μM) initially for 30 min and then by co-application with RYA for 30 min prevented the enhanced potentiation and persistence of E-LTP (gray squares, n = 7). **(B)** Experimental design same as of (A), but ZIP was applied initially for 30 min and then by co-application with AP-5 (black square) prevented enhanced potentiation of E-LTP similar to that of (A) (gray diamonds, n = 7). **(C)** Summary bar graph showing differences in the percentage of S1 potentiation at -30, 60, 90, 120, and 360 min after the induction of E-LTP. **(D, E)** Priming of E-LTP by bath application of RYA (D) or 2xTBS (E) in presence of myr-ZIP (1 μM) prevented tagging interactions within the interval of 240 min (D, n = 7; E, n = 7). (**F**) Summary bar graph showing differences in the level of S1 potentiation at 8 h between primed STC conditions (Figure 3. 3B and 3C) and primed with myr-ZIP inhibited STC conditions (D, E). Symbols and traces in (A, B) and (D, E) as in Figure 3. 1 and Figure 3. 3 respectively. $^{**}P < 0.01$; $^{***}P < 0.001$ by *U* test.

3.1.4 Identity of Synaptic Tag in Primed Synaptic Tagging and Capture

In conventional STC, synaptic tag setting of LTP in the CA1 apical dendrite compartment is mediated by CaMKII (Sajikumar et al., 2007; Redondo et al., 2010). Synaptic tag are characterized by relatively short time-course lasting only 60 min under *in vitro* conditions, after which it undergoes degradation probably by process of dephosphorylation (Frey and Morris, 1998a), therefore limiting the effective time window for STC. Since priming of E-LTP by RyR or synaptic activation of group 1 mGluR extends the duration of synaptic tags up to 5 h, the next question was to investigate by what means primed E-LTP maintains its synaptic tag for such a prolonged time period without degradation. To check whether CaMKII still mediates the synaptic tag setting process in primed E-LTP as the same in non-primed E-LTP, CaMKII inhibitor, KN-62 (5 μM), was bath applied during RYA priming and then alone for the next 60 min (thus a total of 90 min KN-62 application). Thirty minutes after RYA application, E-LTP was induced in S1 in the presence of KN-62, 4 h after which L-LTP was induced in S2 (Figure 3. 7A). It was reported previously that KN-62 at a dose of 5 μM could effectively reset the synaptic tag, thus interfering STC (Sajikumar et al., 2007). Here, surprisingly, it had no effect on the process of primed STC although its application decreased the potentiation of primed E-LTP (Figure 3. 7A), in contrast to its effectiveness in blocking conventional STC reported previously (Sajikumar et al., 2007). Significant potentiation was initially observed up to 220 min relative to the pretetanization (Wilcoxon test, $P = 0.04$), and then it regained gradually to a statistically significant level after the induction of L-LTP in S2 from 285 min onwards (Wilcoxon test, $P = 0.02$). To check the possibility that CaMKII would dominate in the tag-setting during the early phase of STC in the RYA-primed condition, the same experiment paradigm was used as that of Figure 3. 7A with the exception that L-LTP was induced in S2 one hour after the induction of E-LTP in S1. Again, KN-62 prevented the facilitation of primed E-LTP, but subsequently the primed E-LTP was gradually transformed into L-LTP, indicating expression of STC (Figure 3. 7B). The potentiation in S1 was statistically significant for an initial 30 min (Wilcoxon test, $P = 0.04$) and then again from 90 min (Wilcoxon test, $P = 0.04$) until 360 min (Wilcoxon test, $P = 0.01$). In both cases, potentials in S2 showed significant increases from the time of tetanization until 480 min later (Wilcoxon test, $P = 0.01$).

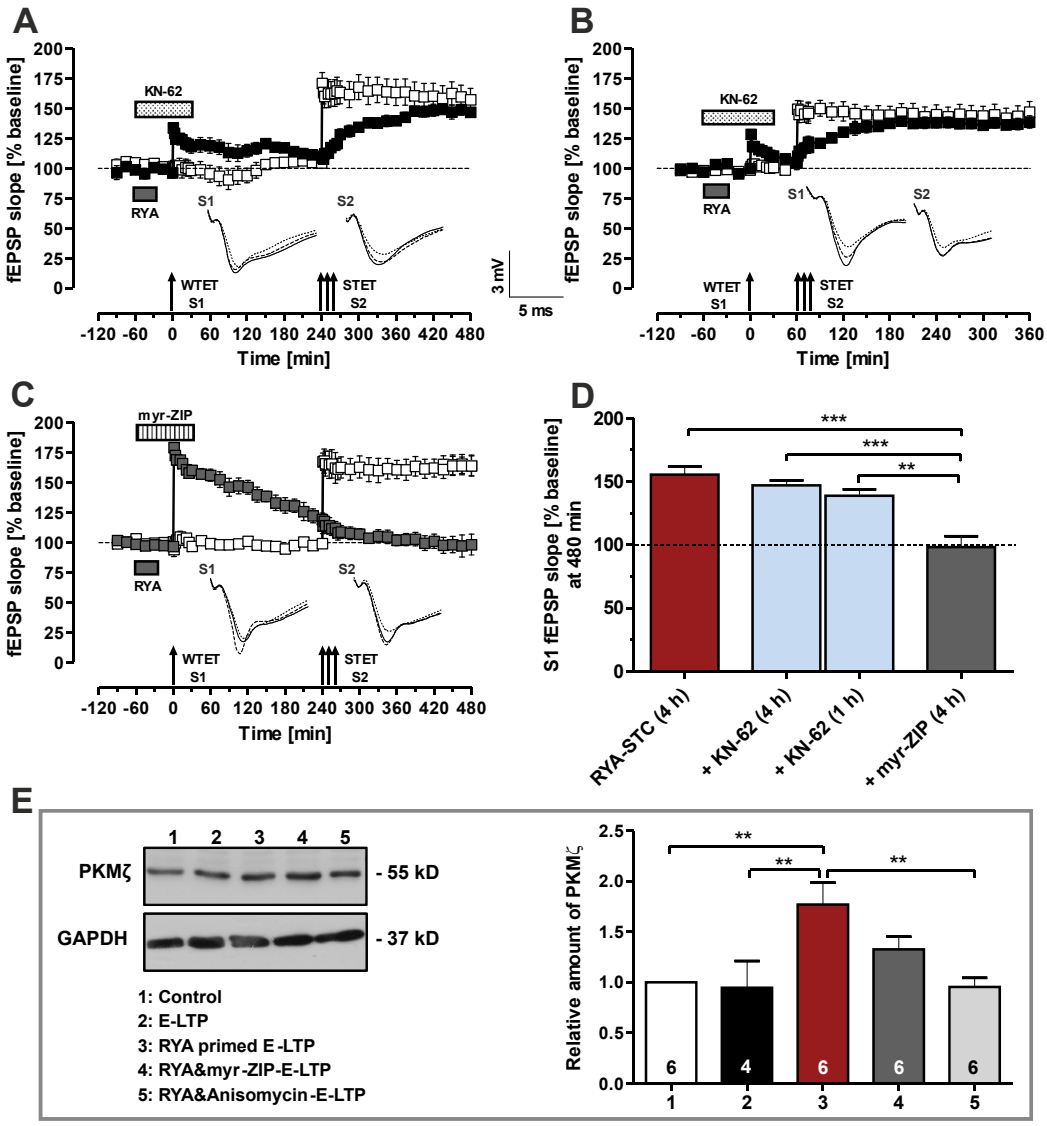


Figure 3. 7 | Metaplasticity alters the molecular mechanisms of synaptic tags.
(A) Priming and order of induction of E-LTP in S1 (black squares) and L-LTP in S2 (white squares) is equivalent to Figure 3. 3B, except that the CaMKII inhibitor KN-62 (5 μM) was applied during priming and then for the next 1 h. CaMKII inhibition did not prevent STC in a late tagging interval of 240 min (n = 7). **(B)** Experiment similar to (A) except that STET was applied to S2 at 60 min. In an early tagging interval of 60 min also, STC was intact (n = 7). **(C)** Application of the PKMζ inhibitor myr-ZIP (1 μM) during RYA priming and during WTET interfered with STC (n = 8). **(D)** Summary bar graph showing differences in the level of S1 potentiation at 8 h presented here in (A–C) and RYA primed STC (Figure 3. 3B). $^{**}P < 0.01$; $^{***}P < 0.001$ by *U* test. **(E)** Western blot (left) and quantification (right) of PKMζ expression revealed a higher expression level of PKMζ in the RYA-primed E-LTP induced group (group 3) in comparison to control conditions (group 1), E-LTP induced group (group 2) and RYA-primed E-LTP in the presence of anisomycin group (Group 5). Although the application of myr-ZIP together with RYR priming and E-LTP inhibited PKMζ function as seen in Figure 3. 7C, it had no effect on the expression rate of PKMζ (group 4). $^{**}P < 0.01$ by one-way ANOVA test. The values of the individual groups were calculated in relation to the control group while tubulin serves as a loading control. The number in each bar represents the number of blots analyzed. Each bar represents mean ± SEM. Symbols and traces as in Figure 3. 3.

Since CaMKII does not mediate the synaptic tag in RYA primed E-LTP, then what other molecular mechanism becomes involved? As it was reported recently that mGluR-dependent priming induces PKMζ as a PRP for STC (Sajikumar and Korte, 2011a), it is reasonable to hypothesize that PKMζ may mediate the setting of synaptic tag in RYA primed E-LTP. To test this, the same experimental design was used as that of in Figure 3. 7A, but instead of KN-62, PKMζ inhibitor myr-ZIP (1 μM) was applied. Intriguingly, PKMζ inhibition prevented RYA-primed E-LTP from expressing STC (Figure 3. 7C), indicating that PKMζ mediates the synaptic tag setting in primed STC. Statistically significant potentiation in S1 was observed only up to 240 min in S1 (Wilcoxon test, P = 0.03), but no significant potentiation was found after the STET to S2, while L-LTP in S2 showed statistically significant potentiation from time point of tetanization until 480 min (Wilcoxon test, P = 0.02). A comparison of S1 potentiation at 480 min in RYA primed STC with these three conditions (Figure 3. 7A–C) is presented in Figure 3. 7D.

In addition to the electrophysiological results, biochemical experiments using western blot analysis of PKMζ provided further evidence that RYA priming trigger the new synthesis of PKMζ which mediates synaptic tag setting. The results showed that there was significantly increased PKMζ expression level in RYA primed E-LTP group (group 3) in comparison to control slices without any stimulation group (group 1), E-LTP without RYA priming group (group 2) or RYA primed E-LTP but protein synthesis inhibitor anisomycin treated group (group 4) (Figure 3. 7E, one-way ANOVA test, P = 0.002). Compared with RYA primed E-LTP group (group 2), PKMζ expression rate was not altered in RYA primed E-LTP but PKMζ inhibitor myr-ZIP treated group (group 3) (Figure 3. 7E, one-way ANOVA test, P > 0.05). This is because myr-ZIP only inhibits the function of PKMζ as shown in Figure 3. 7C and Figure 3. 8A but not the expression level of it, in consistent with earlier study (Sajikumar and Korte, 2011a).

PKMζ is a well established PRP in the conventional STC, to check whether PKMζ also acts as a PRP in the RYA-primed STC, myr-ZIP (1 μM) was bath applied 30 min after the establishment of RYA-primed STC. As shown in Figure 3. 8A and B, PKMζ not only prevented the transformation of RYA primed E-LTP into L-LTP in S1, but also deteriorated the potentiation of L-LTP in S2, suggesting PKMζ also acts as a PRP in primed STC. Statistically significant potentiation in S1 was observed only up to 165 min in S1 (Wilcoxon test, P = 0.01).

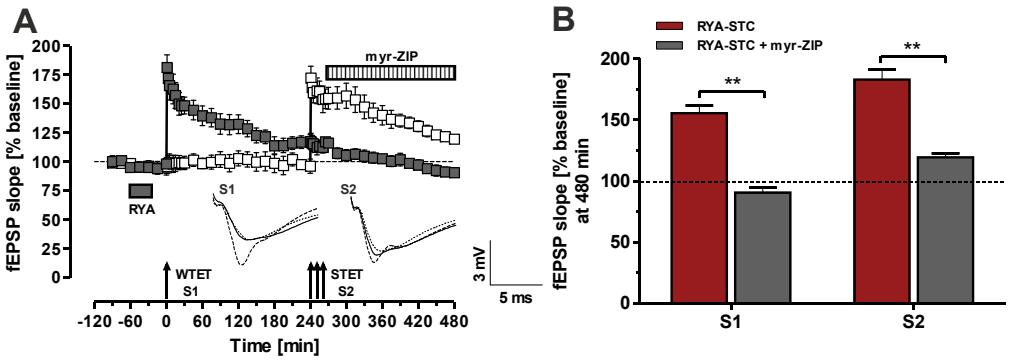


Figure 3. 8 | Identify of PRP in RYA primed STC.
(A) The experimental design was same as of Figure 3. 3C, except that ZIP was applied 30 min after the induction of L-LTP and up to the end of the recordings. PKMζ inhibition not only prevented the maintenance of L-LTP in S2 (gray squares) but also the captured processes of primed E-LTP (white squares) (n = 7). **(B)** Bar graph showing the potentiation of S1 and S2 at 8 h in RYA primed conditions (Figure 3. 3B) is significantly higher than the RYA primed STC but myr-ZIP inhibited conditions (A). $^{**}P < 0.01$ by *U* test. Symbols and traces as in Figure 3. 3.

Taken together, in this part, these data strongly indicates that in contrast to the normal STC, CaMKII is not involved in the tag setting process of RYA-primed STC, but instead PKMζ mediates the tag setting in this primed condition. In addition, PKMζ is a PRP in primed STC, as the same in conventional STC.

3.1.5 Priming Creates Stable Synaptic Tags

Previous studies showed that both LTP and synaptic tag can be reset by depotentiation (DP) in a time-dependent manner. For instance, DP elicited by 1 Hz, 250 pulses 5 min after E-LTP induction in hippocampal CA1 *in vitro* could effectively reset the synaptic tag that mediated by CaMKII, leading to a reversal of E-LTP and absence of its subsequent STC (Sajikumar and Frey, 2004a). However, it was reported earlier that local protein synthesis could provide synaptic immunity against DP (Woo and Nguyen, 2003b). Since RyR or synaptic mGluRs activation primes LTP through *de novo* protein synthesis of PKMζ, it is very likely that primed E-LTP and its STC show synaptic immunity to DP. To test this possibility, the same experimental design was used as that of in Figure 3. 3B or C with the exception that DP, i.e. LFS (1 Hz, 250 pulses) was applied to synaptic input S1 five min after the induction of RYA or 2xTBS primed E-LTP. Intriguingly, in contrast with the conventional E-LTP which is completely reversed by DP (Sajikumar and Frey, 2004a), either RYA or 2xTBS primed E-LTP was only partially but not completely reversed by LFS for a transient 10 min, showing synaptic immunity to DP (Figure 3. 9A and B). After the induction of L-LTP in S2 at 240 min, the potentials in S1 gradually regained to a stable L-LTP lasting 8 h, still expressing STC (Figure 3. 9A, B and E). In both cases, statistically

significant potentiation in S1 was shown from the time point of E-LTP induction until 480 min (Wilcoxon test, $P = 0.01$). Potentiation of S2 was statistically significant from the time point of L-LTP induction until 480 min (Wilcoxon test, $P = 0.01$).

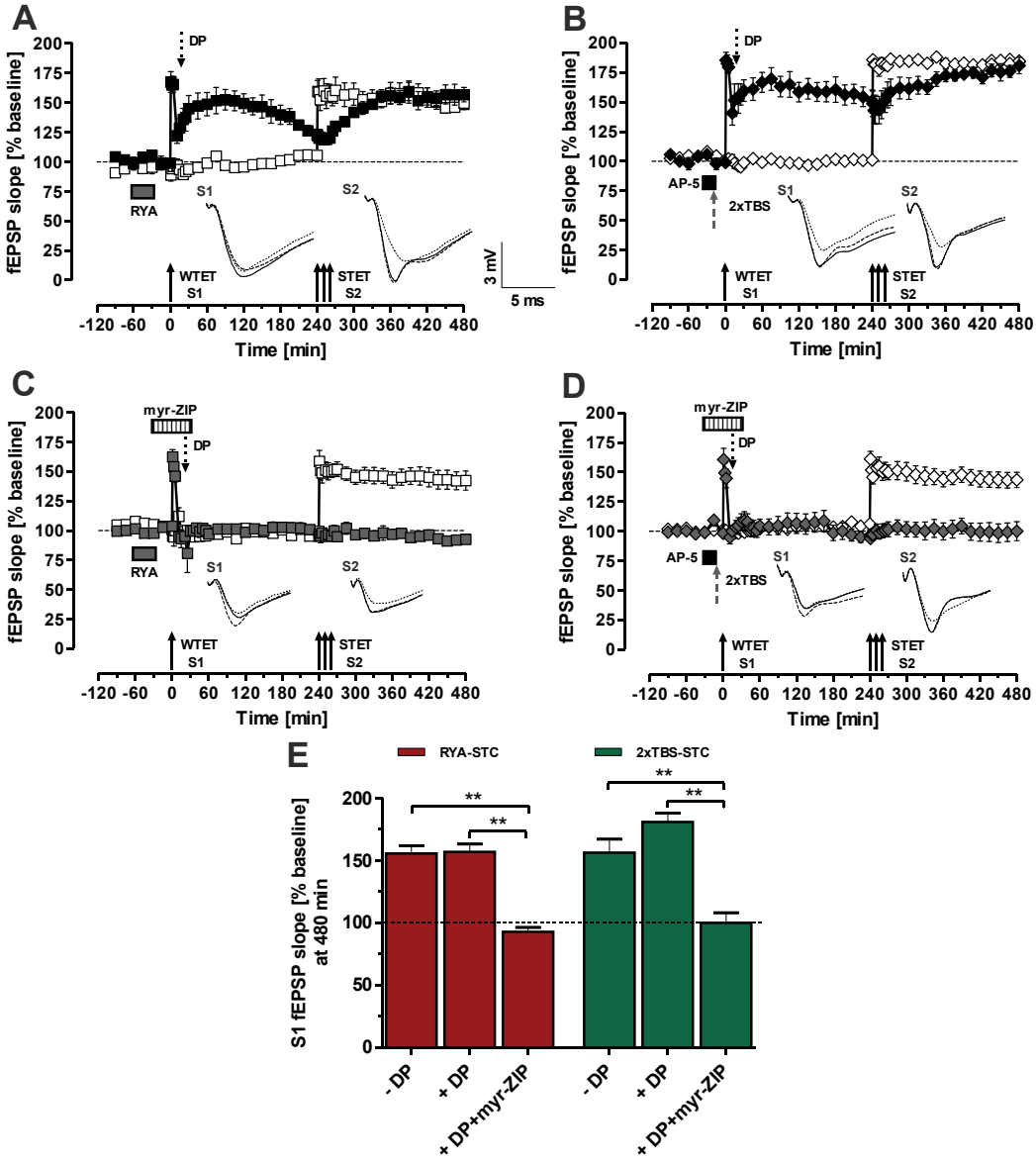


Figure 3. 9 | Priming creates stable synaptic tags.
(A, B) DP (1 Hz, 250 pulse LFS) was applied 5 min after the induction of RYA (A) or synaptically primed E-LTP (B). Primed E-LTP was resistant to DP (black squares in A or black diamonds in B), leaving the synaptic tag intact which results in effective STC (A, n = 6; B, n = 7). **(C, D)** The experimental design was same as that of (A) and (B), with the exception that DP stimulation was delivered during the inhibition of PKMζ by myr-ZIP. In both cases, the depotentiated E-LTP was unable to regain its initial potentiation and subsequent tagging (gray squares, C, n = 7; gray diamonds, D, n = 7). **(E)** Summary bar graph of S1 potentiation at 8 h shows DP had no effect on RYA or 2xTBS primed STC (Figure 3. 3B, C). In contrast, DP application in the presence of myr-ZIP led to a significant decreased potentiation of S1 at 8 h in the primed STC (Figure 3. 9C, D). $^{**}P < 0.01$ by *U* test. Symbols and traces as in Figure 3. 3. In addition, downward dotted arrow represents the time point of DP stimulation.

Since the priming process is able to change the synaptic tag setting machinery from a CaMKII mediated one to a PKMζ mediated one, it is important to check whether DP stimulation during PKMζ inhibition could reset the synaptic tag and thus interfere with STC. To test this, the same experimental paradigm was used as that of Figure 3. 9A and B but myr-ZIP (1 μM) was bath applied 30 min before and 30 min after E-LTP induction, thus WTET and DP were delivered in the presence of myr-ZIP. Intriguingly, DP 5 min after WTET in S1 completely reversed RYA or 2xTBS primed E-LTP and no STC was observed after the delivery of STET in S2 (Figure 3. 9C–E). Primed E-LTP in S1 showed statistically significant potentiation only up to 5 min after its induction (*U* test, $P = 0.01$; Wilcoxon test, $P = 0.01$), while L-LTP in S2 showed statistically significant potentiation from time point of induction onwards until 480 min (Wilcoxon test, $P = 0.01$ in both Figure 3. 9C and D).

Taken together, RYA- and 2xTBS-primed E-LTP are resistant to DP and its effects on late associativity of STC, which is in contrast with non-primed E-LTP. However, PKMζ inhibition during DP blocks both of the two priming effects, thus restoring the sensitivity of the LTP and STC to DP. These data indicate PKMζ mediated synaptic tag is a stable one that is resistant to reversal.

3.2 Metaplasticity Prevents Synaptic Plasticity Deficits in an Alzheimer's Disease Mouse Model

In AD, synaptic plasticity deficits in the hippocampus are considered to be the best neurobiological correlate underlying memory loss. Motivated by the accumulating findings that RyR priming has substantial effects on functional synaptic plasticity, the current work investigated whether inducing metaplasticity through RyR activation in the hippocampus of APP/PS1 mice could prevent the dysregulated synaptic plasticity.

3.2.1 Effects of RyR Priming on L-LTP in APP/PS1 Mice

It is well established that synaptic plasticity such as LTP is impaired in the hippocampus of various AD mouse models including the APP/PS1 mice (Chapman et al., 1999; Gong et al., 2004; Trinchese et al., 2004; Ma et al., 2010). To test L-LTP in APP/PS1 mice in the current study, a control L-LTP was firstly performed in WT mice. After a stable baseline recording of 60 min in both synaptic input S1 and S2, STET was applied to S1, which resulted in L-LTP that lasted for 240 min (Figure 3. 10A). Control stimulation of an independent synaptic input S2 of the same neuronal population revealed stable potentials

during the whole recording period. Statistically significant potentiation was observed in S1 from the time point of L-LTP induction until 240 min (Wilcoxon test, $P = 0.01$; U test, $P = 0.002$). Next, L-LTP was investigated in APP/PS1 mice. In consistent with previous findings (Chapman et al., 1999; Gong et al., 2004; Trinchese et al., 2004; Ma et al., 2010), L-LTP was impaired in APP/PS1 mice, resembling that of an E-LTP, while the control synaptic input S2 was stable during the whole experimental session (Figure 3. 10B). Statistically significant potentiation was observed in S1 only up to 155 min (Wilcoxon test, $P = 0.01$) or 120 min (U test, $P = 0.04$) after the induction of L-LTP.

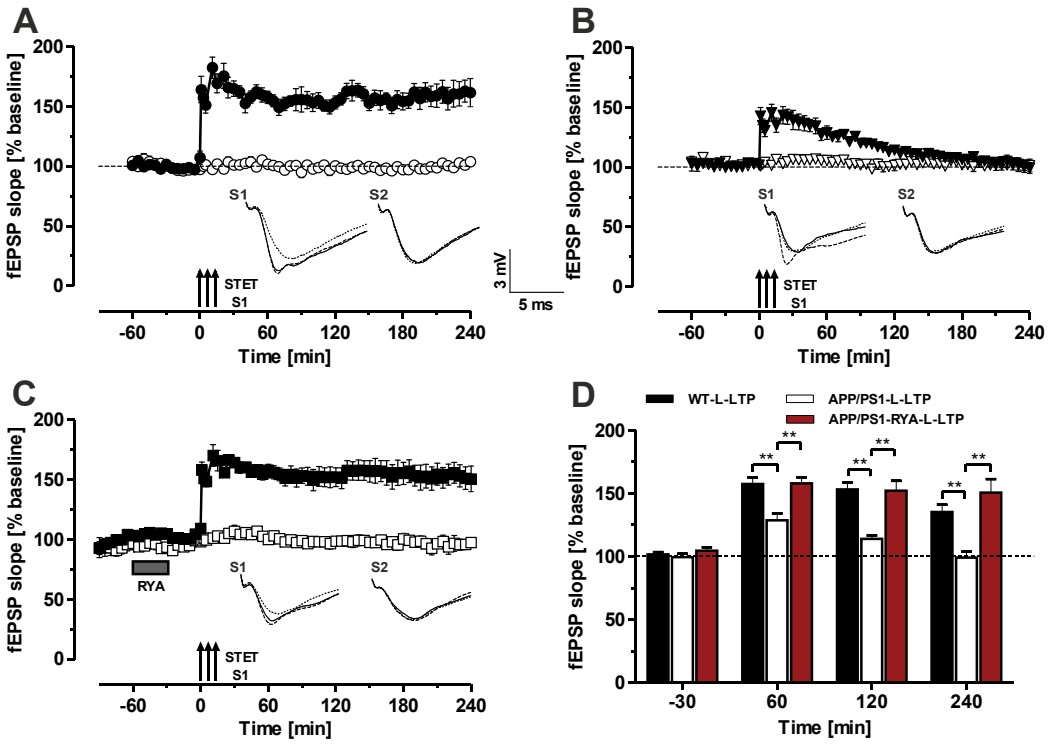


Figure 3. 10 | RyR priming rescues the impaired L-LTP in APP/PS1 mice.
(A) A typical L-LTP induced by strong tetanus (STET, arrows) in S1 (black circles) in WT mice. White circles represent a control stimulated synaptic input S2 which was relatively stable (n = 7). **(B)** The same as that of (A), but STET was applied to S1 to induce L-LTP in APP/PS1, which resulted an LTP lasting less than 120 min (black triangles) without effecting the control input S2 (white triangles) (n = 7). **(C)** Priming of the hippocampal slices of APP/PS1 mice via bath application of RyR agonist RYA (gray rectangle, 10 μM) for 30 min and then washout for 30 min before the induction of L-LTP in S1 significantly increased both the induction and persistence of L-LTP potentiation in S1 (black squares). Control stimulation of S2 (white squares) revealed relatively stable potentials for the time course investigated (n = 8). **(D)** Summary bar graph represents the differences in the percentage of S1 potentiation at -30 min, 120 min and 240 min after the induction of L-LTP between the three different conditions presented in (A–C). The asterisk in 60, 120 and 240 min represents statistically significant potentiation with the compared group (**$P <$ 0.01 by U test). Triplets of arrows represent strong tetanization (STET) applied for inducing L-LTP. Insets in each graph represent typical fEPSP traces recorded from synaptic input S1 and S2 thirty before (dotted line), 30 min after (broken line), and 240 min after (full line). All data are plotted as mean ± SEM. Error bars indicate SEM. Calibration bar for all analog sweeps: 3 mV/5 ms.

To probe the priming effect of RyR activation by its agonist ryanodine (RYA, 10 μM) on L-LTP in APP/PS1 mice, the slices were primed by a 30 min bath-application of RYA, washed out 30 min before the STET in S1 for L-LTP induction. As shown in Figure 3. 10C, RYA priming significantly increased the induction and persistence of L-LTP in APP/PS1 mice without affecting its control input S2, leading to a normal L-LTP as that of in WT mice. Statistically significant potentiation was observed in S1 from the time point of L-LTP induction until 240 min (Wilcoxon test, $P = 0.02$; *U* test, $P = 0.003$). RYA priming restored the impaired L-LTP in APP/PS1 mice to WT level, and the RYA primed L-LTP showed significantly enhanced potentiation up to 240 min compared with non primed-L-LTP in APP/PS1 mice (Figure 3. 10D) (*U* test, $P < 0.01$). In short, these data reveal that L-LTP is impaired in APP/PS1 mice resembling that of an E-LTP, while priming stimulation of RyR could reverse the impaired L-LTP.

3.2.2 Effects of RyR Priming on Synaptic Tagging and Capture in APP/PS1 Mice

Since L-LTP is impaired in APP/PS1 mice, it is intriguing to check the late associativity process of STC. Two-pathway experiments of the "strong-before-weak" (A strong tetanization to one synaptic input followed by a weak tetanization to an independent second synaptic input) protocol was used for the investigation. Firstly, STC was performed in WT mice as previously reported (Redondo et al., 2010b; Martin and Kosik, 2002), a STET protocol for inducing L-LTP was delivered to S1, and 10 min after the last 100 Hz train, a WTET was applied to S2 to induce E-LTP. Here L-LTP was observed in both S1 and S2 lasting 4 h (Figure 3. 11A), indicating the establishment of STC in WT mice. Statistically significant potentiation was found up to 240 min in both S1 and S2 (Wilcoxon test, $P = 0.01$). Next, STC was studied in APP/PS1 mice by using the same experimental paradigm as that of in the WT mice. Surprisingly, STC was not expressed in APP/PS1 mice as the potentiation in both S1 and S2 decayed to the baseline levels within 2 h (Figure 3. 11B). Statistically significant potentiation was observed only up to 150 min (Wilcoxon test, $P = 0.01$) in S1, and up to 110 min (Wilcoxon test, $P = 0.04$) in S2.

It was reported previously that STC can be regulated by a prior RyR-activation dependent mechanism (Sajikumar et al., 2009; Li et al., 2014). Thus, it is of great interest to test whether priming stimulation by RyR activation could have any effects on STC in APP/PS1 mice. To test this, the hippocampal slices of APP/PS1 mice were primed by a 30 min bath-administration of RYA (10 μM), beginning 30 min before a STET to S1 for L-LTP induction and 60 min before a WTET to S2 for E-LTP induction (Figure 3. 11C).

Intriguingly, both S1 and S2 showed L-LTP lasting at least 4 h of the recoding period, thus expressing STC (Figure 3. 11C and D). Statistically significant potentiation were found in both S1 and S2 up to 240 min (Wilcoxon test, P = 0.01). These data reveal that late associativity process of STC is absent in the neural networks of APP/PS1 mice, while metaplasticity though RyR activation re-establishes it.

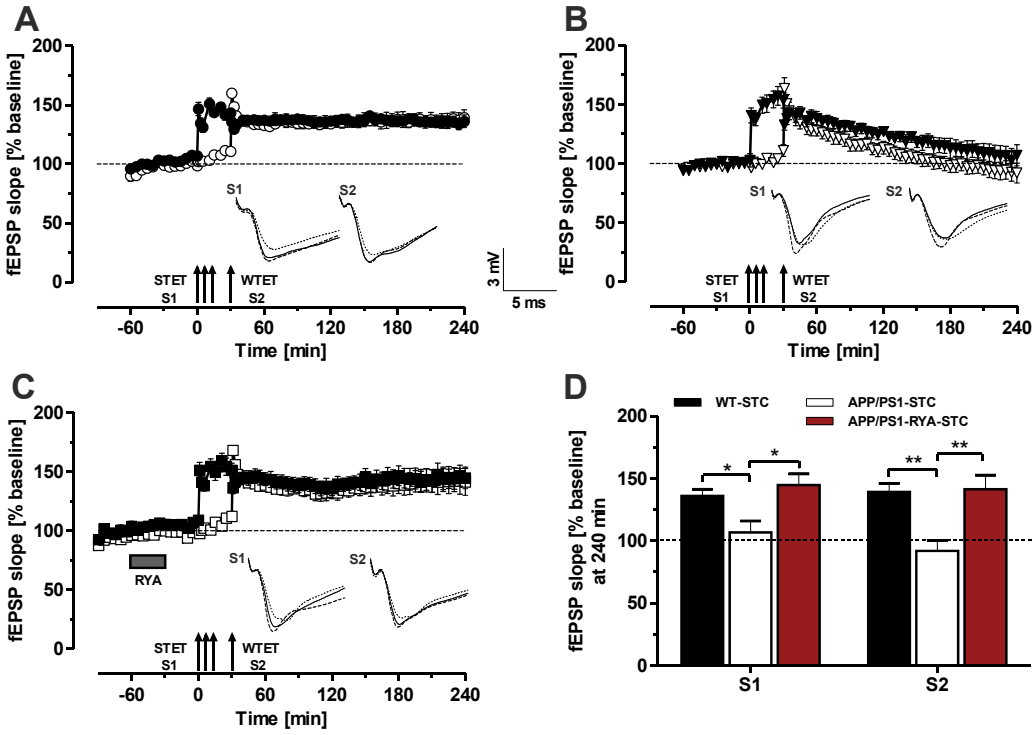


Figure 3. 11 | RyR priming enables establishment of STC in APP/PS1 mice.
(A) Control experiments showing STC induced by a "strong-before-weak" protocol in WT mice. Induction of L-LTP by WTET in S1 (black circles) was followed by E-LTP induced by WTET (single arrow) in S2 (white circles). Here E-LTP was transformed into L-LTP, showing STC (n = 7). **(B)** Experimental design was similar to (A) but STC was induced in APP/PS1 mice, potentiation of both S1 (black triangles)) and S2 (white triangles) returned to baseline levels within 180 min, indicating impaired STC (n = 8). **(C)** As the same in (B) except that RYA (10 μM) was bath applied for 30 min and then washed out for 30 min. Here not only L-LTP in S1 was rescued (black squares), but also E-LTP in S2 was transformed into L-LTP in S2 (white squares), expressing STC (n = 7). **(D)** Bar graph showing differences in the level of potentiation of S1 and S2 after the induction of L-LTP or E-LTP respectively, at 240 min between the three different conditions presented in (A–C). Asterisks indicate significant group differences in potentiation (*P < 0.05; **P < 0.01 by *U* test). Symbols/traces are as in Figure 3. 10. In addition, single arrow represents weak tetanization (WTET) applied for inducing E-LTP.

3.2.3 Effects of RyR Priming on Cross-capture in APP/PS1 Mice

Previous studies have shown that soluble Aβ from several preparations could facilitate LFS induced mGluR-LTD both *in vivo* and *in vitro* (Kim et al., 2001; Cheng et al., 2009) but has no effect on NMDAR-LTD (Wang et al., 2002; Raymond et al., 2003; Li et al., 2009). Thus, it is of interest to investigate L-LTD and its late associativity of cross-capture (i.e.,

interaction between L-LTD and E-LTP) in APP/PS1 mice. In a control set of experiments, L-LTD in WT mice was induced by a SLFS to S1, which resulted in L-LTD lasting 4 h without altering the potentials of the control input S2 (Figure 3. 12A). The potentials in S1 was statistically significantly decreased 21 min after the induction of L-LTD till the end of recording point 240 min (Wilcoxon test, $P = 0.005$; *U* test, $P = 0.002$). In the next series of experiments, L-LTD in APP/PS1 mice was tested. As shown in Figure 3. 12B, L-LTD seems normal in APP/PS1 mice, which is consistent with the findings that Aβ has no effect on NMDAR-LTD induced by stronger LFS protocols (i.e., 900-pulse) (Wang et al., 2002; Raymond et al., 2003; Li et al., 2009). Statistically significant depression was maintained up to the end of the recording period of 240 min after SLFS (Wilcoxon test, $P = 0.01$; *U* test, $P = 0.01$), while the potentials of control input S2 remained stable during the recording session.

For checking cross-capture in APP/PS1 mice, a control cross-capture was initially performed in WT mice. To induce cross-capture, SLFS was applied to S1 for L-LTD induction and 45 min later, WTET was delivered to S2 for E-LTP induction. Here not only L-LTD in S1 was stable during the recording session, but also E-LTP in S2 was converted into L-LTP (Figure 3. 12C), showing cross-capture. Statistically significant depression or potentiation was observed in S1 and S2 respectively up to 240 min (Wilcoxon test, $P = 0.01$). The same cross-capture paradigm was then applied to hippocampal slices of APP/PS1 mice to induce cross-capture. As shown in Figure 3. 12D, although L-LTD seems not altered in APP/PS1 mice (LTD until 240 min, Wilcoxon test, $P = 0.01$), E-LTP in S2 could not benefit the PRPs from it and thus cannot be transformed into L-LTP (statistically significant potentiation was only maintained until 140 min, Wilcoxon test, $P = 0.03$), suggesting no cross-capture in APP/PS1 mice.

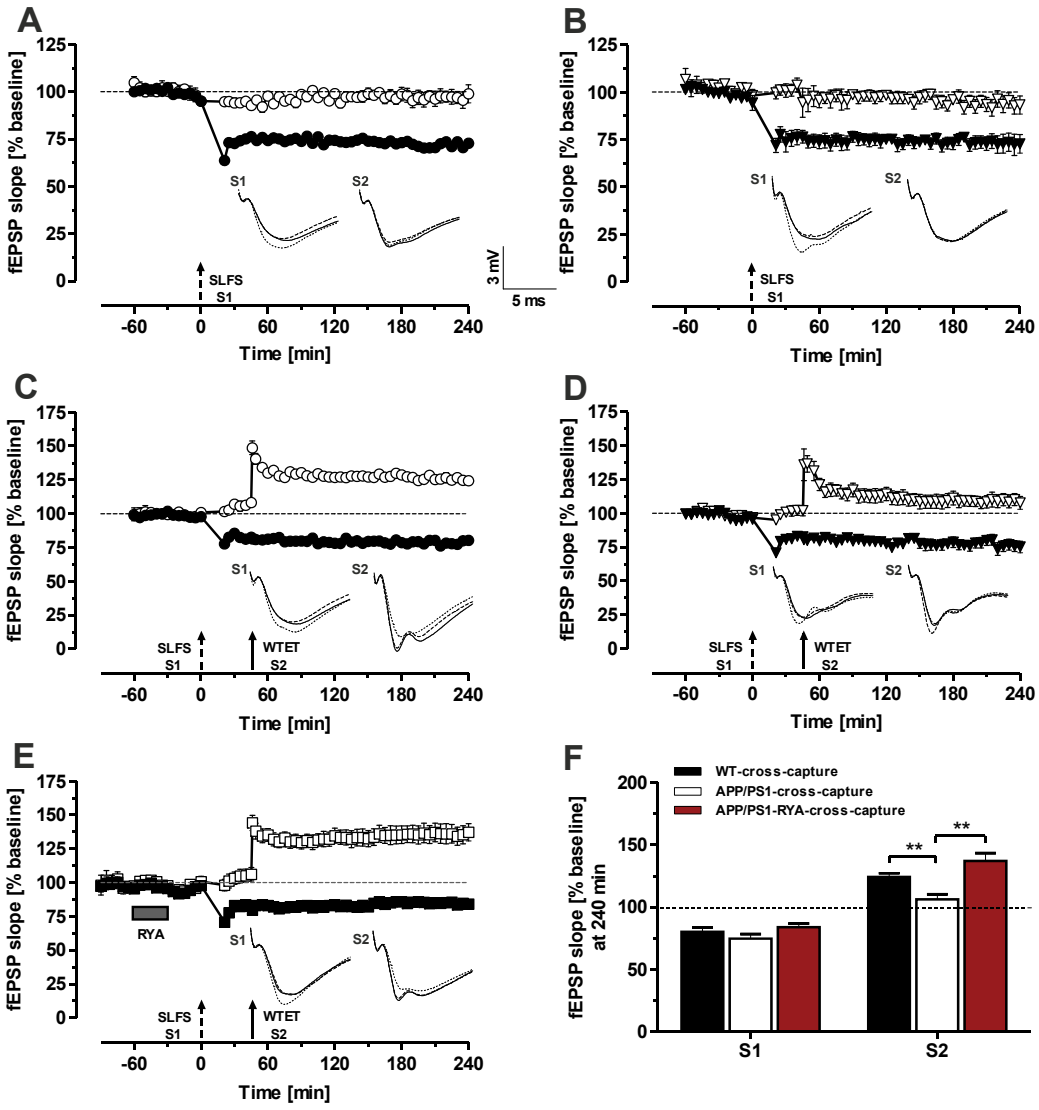


Figure 3. 12 | RyR priming enables formation of cross-capture in APP/PS1 mice.
(A) In WT mice, a strong low frequency stimulation (SLFS, broken arrow) applied to synaptic input S1 resulted in a significant L-LTD (black circles) lasting for 4 h, control input S2 (white circles) that received test pulses were stable during the whole recording period (n = 11). **(B)** The same as that of (A), except that L-LTD was induced in APP/PS1 mice (n = 8). **(C)** In WT mice, E-LTP by a WTET in S2 (open circles) can be converted to L-LTP provided that L-LTD was induced by SLFS in S1 (black circles) 45 min prior to the induction of E-LTP, showing cross-capture (n = 7). **(D)** The same experimental paradigm as that of (C) with the exception that cross-capture was studied in APP/PS1 mice. Here E-LTP failed to be transformed into L-LTP in S2 (white triangles), indicating no cross-capture in APP/PS1 mice (n = 7). **(E)** Priming stimulation with RYA (10 µM) for 30 min enabled the establishment of cross-capture in APP/PS1 mice, as E-LTP in S2 was transformed into L-LTP (black squares) while L-LTD in S1 (white squares) was not affected (n = 9). **(F)** Summary bar graph showing differences in the level of S2 potentiation at 240 min between the three conditions presented in (C–E), and no differences of the level of S1 depression at 240 min. Asterisks indicate significant group differences in potentiation (**P < 0.01 by *U* test). Symbols/traces are as in Figure 3. 10. In addition, single broken arrow represents strong low frequency stimulation (SLFS) applied for inducing L-LTD.

Since RyR priming regulates STC in APP/PS1 mice, and importantly it triggers new PKMζ synthesis as a PRP to promote STC (Li et al., 2014), it is reasonable to test RyR priming on cross-capture in APP/PS1 mice. The same cross-capture paradigm was used as that in Figure 3. 12C and D, except that a 30 min bath-application of RYA (10 μM) begun 30 min prior to the induction of L-LTD in S1. Intriguingly, RYA priming restored the cross-capture in APP/PS1 mice, as E-LTP in S2 was transformed into L-LTP lasting till the end of recordings at 240 min (Figure 3. 12E and F) (Wilcoxon test, $P = 0.007$), while S1 still shows L-LTD (statistically significant depression was maintained until 240 min, Wilcoxon test, $P = 0.007$).

3.2.4 RyR Priming Exerts its Effect through Protein Synthesis

It was reported previously that prior activation of group I mGluRs or RyRs facilitates the subsequent LTP though protein synthesis mechanism (Mellentin et al., 2007; Sajikumar and Korte, 2011). To check whether it is the same case in the pathological conditions of APP/PS1 mice, the same experimental design was used as that in Figure 3. 10C, except that protein synthesis inhibitor anisomycin (ANI; 25 μM) was co-applied during RYA priming for 30 min. As shown in Figure 3. 13A, protein synthesis inhibition did not affect the initial induction of subsequent LTP but caused it to decay rapidly to the baseline levels within 2 h after STET. Statistically significant potentials in S1 was only observed up to 125 min (Wilcoxon test, $P = 0.04$; *U* test, $P > 0.05$). The persistence of RYA primed APP/PS1-L-LTP in the presence of anisomycin was similar to the APP/PS1-L-LTP in non-primed slices (Figure 3. 13B). Thus, new proteins were synthesized during RYA priming and are mandatory to reverse the impaired L-LTP in APP/PS1 mice. Since priming stimulation of RyR reverses the impaired L-LTP in APP/PS1 mice through protein synthesis mechanism, it is intriguing to test whether this metaplastic upregulation of protein synthesis underlies the rescue of STC in APP/PS1 mice. As shown in Figure 3. 13C and D, application of anisomycin (ANI; 25 μM) during RYR priming prevented the RYA rescued STC in APP/PS1 mice because L-LTP in both S1 and S2 were absent. Statistically significant potentiation was found only up to 125 min (Wilcoxon test, $P = 0.01$) in S1 and 85 min (Wilcoxon test, $P = 0.04$) in S2. Together, these results show that priming stimulation of RyR triggers *de novo* protein synthesis, thereby reversing the impaired L-LTP and STC in APP/PS1 mice.

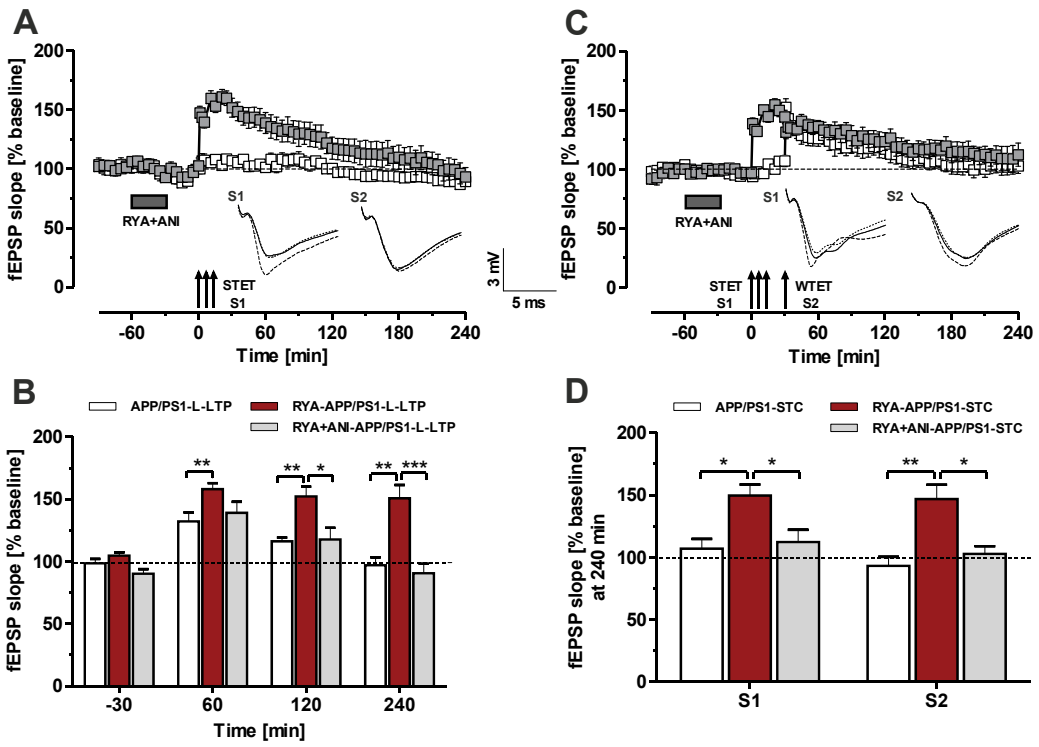


Figure 3. 13 | RyR priming requires protein synthesis in APP/PS1 mice.
(A) Application of protein synthesis inhibitor anisomycin (ANI; 25 μM) during RYA priming abolished the priming effect of RYA in APP/PS1 mice, leading to a decayed LTP (gray squares) without affecting the baseline potentials in S2 (white squares) (n = 8). **(B)** Summary bar graph represents the differences in the percentage of S1 potentiation at -30 min, 60 min, 120 min and 240 min after the induction of L-LTP between the three different conditions presented in (A) and Figure 3. 10B, C. **(C)** Similarly, when ANI (25 μM) was coapplied with RYA for 30 min, no STC was observed in APP/PS1 mice as both input S1 and S2 showed declined LTP (n = 7). **(D)** Bar graph showing differences in the level of potentiation of both S1 and S2 after the induction of L-LTP or E-LTP respectively, at 240 min between the three different conditions presented in (C) and Figure 3. 11B, C. Symbols/traces are the same as in Figure 3. 11. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by *U* test.

3.2.5 RyR Priming Triggers PKMζ Synthesis in APP/PS1 Mice

Since RyR priming triggers protein synthesis to facilitate the subsequent LTP, and compelling evidence showed that either group 1 mGluR or RyR activation leads to PKMζ synthesis as a PRP to regulate STC (Sajikumar and Korte, 2011a; Li et al., 2014), it was intriguing to probe whether RYA priming reverses the impaired synaptic plasticity in APP/PS1 mice through the new synthesis of PKMζ. To test this, myr-ZIP (1 μM), was initially applied for 15 min before co-applation with RYA (10 μM) for another 30 min. As shown in Figure 3. 14A and C, the rescued L-LTP by RyR priming in APP/PS1 was blocked by myr-ZIP application without having any effect on the control input S2. Statistically significant potentiation in S1 was observed only up to 135 min after the induction of RYA primed L-LTP (Wilcoxon test, $P = 0.04$; U test, $P = 0.01$). Control

experiments using an inactive scrambled control peptide of myr-ZIP, scr-ZIP (1 μM), showed no inhibitory effects on the primed L-LTP in APP/PS1 mice (Figure 3. 14B and C). Statistically significant potentiation in S1 was observed after the induction of RYA primed L-LTP until 240 min (Wilcoxon test, $P = 0.02$; U test, $P = 0.008$).

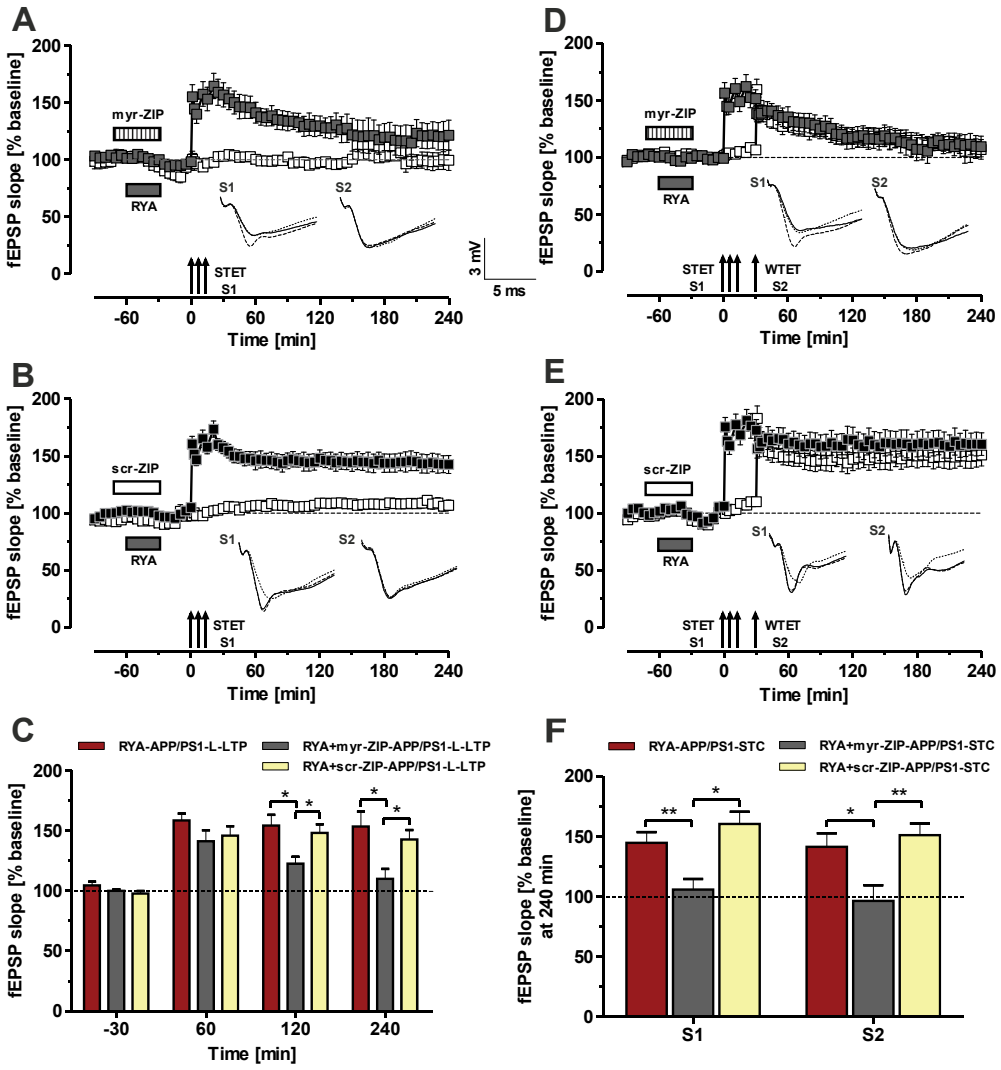


Figure 3. 14 | RyR priming exerts its effects through PKMζ synthesis.
(A) Administration of PKMζ inhibitor myr-ZIP (1 μM) alone for 15 min and then together with RYA for 30 min attenuated the rescued L-LTP in S1 in APP/PS1 mice (gray squares), whereas the potentials of the control pathway S2 (white squares) remained stable throughout the recording period (n = 7). **(B)** Control experiments with control peptide scr-ZIP (1 μM) showed no effects on the RYA primed L-LTP in APP/PS1 mice (n = 6). **(C)** Summary bar graph represents the differences in the percentage of S1 potentiation at -30 min, 60 min, 120 min and 240 min after the induction of L-LTP between the three different conditions presented in (A, B) and Figure 3. 10C. **(D)** PKMζ blockade by myr-ZIP (1 μM) 15 min before and during RYA priming prevented not only the maintenance of L-LTP in S1 (gray squares) but also the transformation of E-LTP into L-LTP in S2 (white squares) in APP/PS1 mice, expressing no STC (n = 7). **(E)** The control peptide scr-ZIP had no effect on primed STC (n = 6). **(F)** Bar graph showing differences in the level of potentiation of both S1 and S2 after the induction of L-LTP or E-LTP respectively, at 240 min between the three different conditions presented in (E, F) and Figure 3. 11C. Symbols/traces are the same as in Figure 3. 11. $*P < 0.05$, $**P < 0.01$ by U test.

As priming RyR activation results in the generation of new PKMζ which can be utilized by the subsequent L-LTP, it is important to test whether the newly generated PKMζ is also mandatory for the primed STC in APP/PS1 mice. Bath-administration of myr-ZIP (1 μM) alone for 15 min and together with RYA (10 μM) for 30 min prevented not only the primed L-LTP in S1 but also the transformation of E-LTP into L-LTP in S2 (Figure 3. 14D and F), reflecting the abolishment of the effect of RyR priming on STC by PKMζ inhibition. Synaptic potentiation showed statistically significant potentials only up to 135 min in S1 (Wilcoxon test, $P = 0.03$) and 115 min in S2 (Wilcoxon test, $P = 0.03$). Control experiments using scr-ZIP showed intact RYA primed STC (Figure 3. 14E and F). Both inputs S1 and S2 showed statistically significant potentials up to the whole recording session of 240 min (Wilcoxon test, $P = 0.02$).

In addition to these pharmacological experiments, biochemical experiments were conducted to check the expression level of PKMζ in APP/PS1 mice and, whether priming stimulation of RyR leads to new PKMζ synthesis. As shown in Figure 3. 15A, compared with WT mice, PKMζ expression level was significantly decreased in the hippocampal CA1 of APP/PS1 mice (t-test, $P = 0.0002$). Strikingly, in APP/PS1 mice, PKMζ level was increased 1 h after RYA primed L-LTP (group 3) in comparison to L-LTP group without RYA priming (group 2) and RYA primed L-LTP but in the presence of anisomycin group (group 4) (Figure 3. 15B) (one-way ANOVA, $P < 0.05$). Although the application of myr-ZIP together with RYA during priming inhibits PKMζ function as shown in Figure 3. 14A and D, it had no effect on the expression rate of PKMζ (group 5, Figure 3. 15B, no statistically significant difference in the expression of PKMζ compared with group 3, $P > 0.05$), similar to previous reports (Sajikumar and Korte, 2011a; Li et al., 2014). The control peptide scr-ZIP had no effects on both the function of PKMζ as shown in Figure 3. 14B and E, and the expression of PKMζ (group 6, Figure 3. 15B, no statistically significant difference in the expression of PKMζ compared with group 3, $P > 0.05$). Collectively, these findings reveal that there is decreased PKMζ expression in the hippocampal CA1 of APP/PS1 mice, while metaplasticity by RyR activation could lead to new PKMζ synthesis, thereby rescuing the impaired L-LTP and STC.

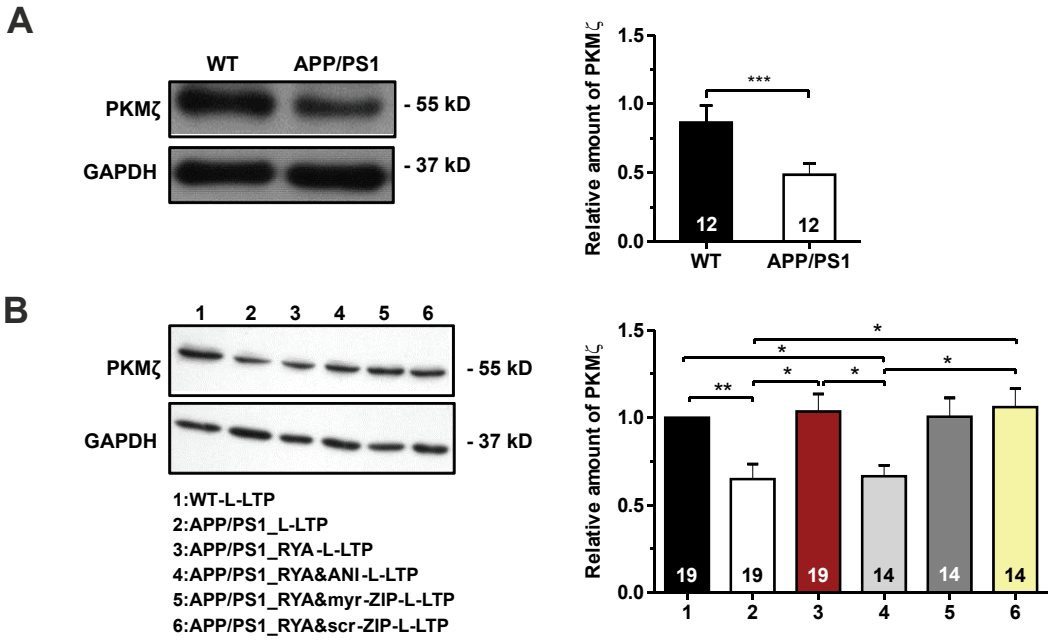


Figure 3. 15 | RyR priming leads to new PKMζ synthesis.
(A) Western blot (left) and quantification (right) of PKMζ protein reduction in APP/PS1 mice normalized to GAPDH. **(B)** Western blot (left) and quantification (right) of PKMζ protein expression revealed a higher expression of PKMζ in APP/PS1 mice 1 h after the induction of RYA-primed L-LTP (group 3) in comparison to non-primed L-LTP group (group 2) and RYA-primed L-LTP in the presence of the anisomycin group (group 4). Although the application of myr-ZIP together with RYR priming inhibited PKMζ function as seen in Figure 3. 14A and D, it had no significant effect on the expression rate of PKMζ (group 5). Likewise, the control peptide scr-ZIP applied in the same way at that of myr-ZIP had no effect on the expression level of PKMζ (group 6). In addition, PKMζ level after L-LTP induction in APP/PS1 mice (group 2) was significantly lower than that in WT mice (group 1). The values of the individual groups were calculated in relation to the control group while GAPDH serves as a loading control. The number in each bar represents the number of blots analyzed. Each bar is presented as mean ± SEM.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by t-test in (A) and one-way ANOVA test in (B).

3.2.6 Identity of PRPs in Primed LTP in APP/PS1 mice

What PRPs are captured by the tagged synapses in RyR primed L-LTP, STC and cross-capture in APP/PS1 mice? In conventional STC, PKMζ is identified as the first LTP-specific PRP (Sajikumar et al., 2005b; Sajikumar et al., 2009; Sajikumar and Korte, 2011a; Li et al., 2014). To test whether a similar mechanism was active in the RyR primed conditions of APP/PS1 slices, PKMζ inhibitor myr-ZIP (1 μM) was bath-applied 60 min after the induction of RYA primed L-LTP till the end of the experiment. As shown in Figure 3. 16A and C, myr-ZIP prevented the primed L-LTP leading to a decayed LTP, as the same as reported previously in the non-primed physiological neural network (Ling et al., 2002; Serrano et al., 2005). Statistically significant potentiation in S1 was observed to 215 min (Wilcoxon test, $P = 0.01$) or 225 min (U test, $P = 0.04$). By contrast, control experiments using scr-ZIP showed normal maintenance of primed LTP (Figure 3. 16B and C).

Statistically significant potentials in S1 was observed during the whole recording session of 240 min (Wilcoxon test, $P = 0.01$; *U* test, $P = 0.003$). In both cases, the control input S2 showed stable potentials during the recording session.

Similarly, the effect of PKMζ inhibition on RYA primed STC were investigated. Bath application of myr-ZIP (1 μM) 30 min after the establishment of RYA primed STC prevented L-LTP maintenance in both synaptic input S1 and S2 (Figure 3. 16D and F), thus no expression of STC. Potentiation in S1 lasted only up to 170 min (Wilcoxon test, $P = 0.02$), whereas in S2 it lasted up to 190 min (Wilcoxon test, $P = 0.003$). Control experiments with scr-ZIP (1 μM) showed normal primed STC (Figure 3. 16E and F). Both inputs S1 and S2 showed statistically significant potentiation after the induction of LTP until the end of the recording period of 240 min (Wilcoxon test, $P = 0.01$). Not only in STC, but also in cross-capture, PKMζ acts as an LTP specific PRP (Sajikumar et al., 2005b). Thus, the next question was to check whether PKMζ was also a PRP in RYA primed cross-capture. To test this, myr-ZIP (1 μM) was bath applied 75 min after the establishment of RYA primed cross-capture. Here, PKMζ inhibition prevented only the transformation of E-LTP into L-LTP in S1, but had no effects on L-LTD in S2 (Figure 3. 16G and H), which is in agreement with previous finding (Sajikumar et al., 2005b). Statistically significant potentiation in S1 was only observed up to 195 min (Wilcoxon test, $P = 0.02$), whereas statistically significant depression was maintained until 240 min (Wilcoxon test, $P = 0.02$). Briefly, these data reveal that RYA primed L-LTP, STC and cross-capture utilizes PKMζ as an important PRP for their consolidation, resembling that of the conventional tagging and capture.

– Legends continue from next page. – **(D)** Continues blockade of PKMζ by myr-ZIP (1 μM) 30 min after the establishment of STC prevented RYA primed STC, as both S1 and S2 decayed to baseline gradually (n = 7). **(E)** Experimental design similar to (C), but scr-ZIP (1 μM) was used, showing no effect on RYA primed STC (n = 7). **(F)** Summary bar graph showing differences in the level of potentiation of S1 and S2 at 240 min between the three different conditions presented in (D, E) and Figure 3. 11C. **(G)** Continuous blockade of PKMζ by myr-ZIP (1 μM) 75 min after the establishment of cross-capture had no effect on L-LTD in S1 (gray squares) but prevented the conversion of E-LTP to L-LTP in S2 (white squares) (n = 7). **(H)** Summary bar graph showing differences in the level of S2 potentiation at 240 min between the three different conditions presented in (G) and Figure 3. 12D and E, while the depression level of S1 at 240 min was not altered. Symbols/traces are as in Figure 3. 11 and Figure 3. 12. *P < 0.05, **P < 0.01 by *U* test.

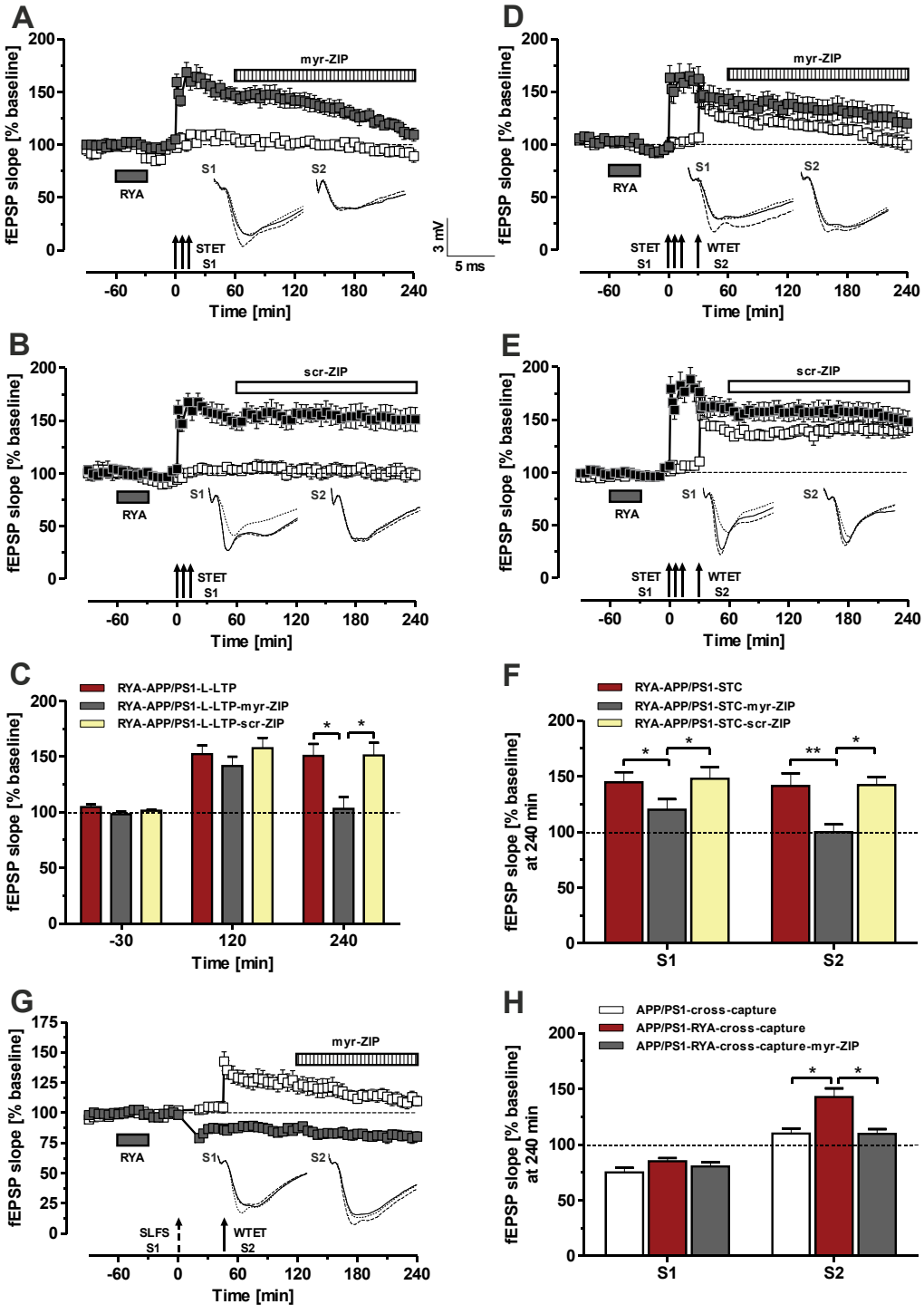


Figure 3. 16 | Identity of PRP in primed L-LTP in APP/PS1 mice.
(A) Continues application of the PKMζ inhibitor myr-ZIP (1 µM) 60 min after induction of L-LTP prevented RYA primed L-LTP (gray squares). Baseline potentials recorded from S2 (white squares) showed stable potentials during the entire recording period (n = 7). **(B)** Experimental design similar to (A) but the scrambled version of myr-ZIP, scr-ZIP (1 µM) was used which had no effect on primed L-LTP (n = 7). **(C)** Summary bar graph represents the differences in the level of potentiation of S1 at -30 min, 120 min and 240 min after the induction of L-LTP between the three different conditions presented in (A, B) and Figure 3. 10C. – Legends continue on previous page. –

4 DISCUSSION

My research has focused on the cellular and molecular mechanisms of how neurons under physiological and pathophysiological conditions adapt their input/output characteristics due to changes in synaptic activity. In this context, metaplasticity is an emerging theme that influences different aspects of functional plasticity (Hulme et al., 2013). Metaplasticity determines LTP/LTD persistence (Abraham and Bear, 1996), creates new synaptic tags for capturing PRPs leading to stabilization of synaptic strength (Sajikumar et al., 2009) and governs the compartmentalization of STC by regulating different functional plasticity factors such as BDNF and PKMζ (Sajikumar and Korte, 2011a). The current study showed that metaplasticity by RyR activation prolongs the synaptic tag duration by switching synaptic tag setting mechanisms from a CaMKII-mediated process (in non-primed STC) to a PKMζ-mediated process (in primed STC). In addition, metaplastic upregulation of PKMζ by RyR activation prevents synaptic plasticity deficits such as L-LTP, STC and cross-capture in an AD mouse model of APP/PS1 mice.

4.1 Metaplasticity and LTP

4.1.1 RyR or mGluR Priming Facilitates LTP

Synaptic plasticity is capable of undergoing adaptive modifications. In particular, the threshold for LTP induction is not static, but varies dynamically according to the recent history of neural activity, as revealed by computational model of BCM theory and experimental evidences (Bienenstock et al., 1982; Abraham and Tate, 1997). Prior group 1 mGluR activation, for instance, lowers the threshold for LTP induction, thus facilitating both the induction and persistence of the subsequent LTP (Bortolotto et al., 1994; Cohen and Abraham, 1996; Raymond et al., 2000). In the present study, priming stimulation of RyR by its agonist RYA (10 μM) facilitates the subsequent E-LTP, resulting in an intermediate form of LTP that is nonetheless still decremental, which is in line with earlier findings (Mellentin et al., 2007; Sajikumar et al., 2009). The RyR or mGluR primed E-LTP is similar to the intermediate form of LTP dependent on group 1 mGluR activation (Raymond et al., 2000), which requires protein synthesis but is transcription independent. This form of LTP was identified in the DG, while the present data indicate that such an intermediate phase of LTP also exists in the CA1 area of hippocampus. In addition, RyR

priming of E-LTP is as effective as that of synaptic mGluR priming, which is quite a good indication that RyR priming is physiologically relevant. However, it seems that in these synaptic mGluR priming experiments the facilitation of E-LTP is slightly higher than that of the pharmacological RyR priming protocol. This can be interpreted in a way that synaptic priming triggers the release of other meuromodulators in addition to glutamate, such as noradrenaline, which is also capable of priming LTP induction (Cohen et al., 1999). Furthermore, both RyR and synaptic mGluR primes LTP homosynaptically or input-specific (i.e., for the same synapses that were primed), as no facilitating effect on the control synaptic input was found. These data here confirm previous findings (Raymond et al., 2000). Of note, it has been reported recently that RyR agonist such as ryanodine or caffeine can facilitate LTP in the ventral hippocampus (VH), but not in the dorsal hippocampus (DH) and these differences are due to the different distribution of RyRs (Grigoryan et al., 2012). However, the current work did not differentiate between DH and VH for experimental reasons.

4.1.2 Mechanisms of RyR or mGluR-Mediated Metaplasticity

It was already demonstrated that the priming effects of mGluR activation are mediated by the activation of PLC (Cohen et al., 1998), the release of Ca^{2+} from intracellular stores (mainly the RyR) and the entry of Ca^{2+} through SOCs in the plasma membrane (Mellentin et al., 2007). Direct transient activation of RyRs leads to the intracellular Ca^{2+} release, supplemented by Ca^{2+} through SOC in the plasma membrane (Mellentin et al., 2007). Ultimately, the elevated Ca^{2+} in the postsynaptic sites triggers the activation of several kinases such as ERK1, ERK2, αCaMKII and PKC, which contribute to the activation of local protein synthesis machinery. The pathway activated by mGluR stimulation leads to local protein synthesis resulting in the activation of the protein kinase mammalian target of rapamycin (mTOR) (Abraham, 2008; Sajikumar and Korte, 2011a). In particular, the synthesis of necessary proteins in close proximity to sites of RyR activation might create a pool of PRPs being kept in reserve for enhancing the persistence of further functional plasticity.

However, the identity and function of the synthesized proteins by RyR priming has yet to be elucidated. One recent study showed that prior mGluRs by its agonist DHPG generates PKMζ as a PRP for the subsequent plasticity events (Sajikumar and Korte, 2011a). In the present study, myr-ZIP inhibition during RYA or synaptic mGluR priming prevents the effects of RyR priming. In addition, biochemical evidences show that there is increased

PKMζ expression in RYA primed hippocampal slices in comparison to non-primed ones. These findings provide compelling evidences that priming RyR activation triggers *de novo* protein synthesis of PKMζ, which has substantial effects on the subsequent plasticity events. This is in line with the observation that intrahippocampal injection of ryanodine (RYA) in rats at concentrations that stimulate RyR-mediated Ca^{2+} release enhances spatial memory formation and consolidation by specifically increasing the RyR subtypes such as RyR2 and RyR3, plus BDNF and PKMζ (Adasme et al., 2011). Therefore, both RyR and mGluR activation primes LTP by producing new protein synthesis of PKMζ. These findings confirm and extend the earlier reports that group 1 mGluR activation couples to nearby protein synthesis machinery from pre-existing mRNA (here PKMζ mRNA) (Raymond et al., 2000; Sajikumar and Korte, 2011a).

4.2 Metaplasticity Prolongs Associativity of Long-term Memory

4.2.1 Metaplasticity Alters the Mechanisms of Synaptic Tag Setting

According to STC, the input-specificity of LTP is achieved by the activation of process- and compartment-specific tag molecules (Sajikumar et al., 2007). It is assumed that protein kinases are involved in the tag setting machinery or being an essential part of the tag complex itself (Martin and Kosik, 2002; Reymann and Frey, 2007). One of these protein kinases that mediates the setting of synaptic tag in conventional STC is CaMKII, which mediates the synaptic tag setting in LTP and notably in the CA1 apical dendrite compartment (Sajikumar et al., 2007; Redondo et al., 2010). In addition, CaMKII is identified as being necessary for behavioral tagging (Moncada et al., 2011). A plasticity-related extracellular protease neuropsin, which is important for STC at apical dendrites, also acts via CaMKII signaling (Ishikawa et al., 2011). In particular, CaMKII mediates the synaptic tag setting in RyR primed STP (Sajikumar et al., 2009). However, the present data show that in contrast to conventional STC, CaMKII is not involved in the synaptic tag setting process in RyR primed STC, neither in the early stage nor the late stage. Instead, PKMζ becomes essential in tagging the synapse in RyR primed STC. Therefore, RyR priming alters the synaptic tagging machinery from a CaMKII-mediated process to a PKMζ-mediated process. This finding might explain how the “life-time” of a synaptic tag is extended in primed E-LTP, as PKMζ is a persistently active kinase which can maintain plasticity up to hours and weeks, much longer than the activation of CaMKII (Sacktor, 2011).

But how does PKMζ extend the duration of synaptic tags in RyR primed E-LTP? CaMKII-mediated synaptic tag lasts only 60 min (Frey and Morris, 1998a; Sajikumar et al., 2007), after which the tag degrades probably due to dephosphorylation. Thus, one possibility is that PKMζ prevents the degradation of the CaMKII-mediated synaptic tag by protecting it from dephosphorylation. Alternatively, PKMζ may act by replacing CaMKII from the tag-setting process. If PKMζ protects the CaMKII-mediated tag from degradation, tagging should still be blocked by CaMKII inhibitor (Sajikumar et al., 2007; Redondo et al., 2010). However, even in the CaMKII-inhibited situation, the primed E-LTP expresses STC both at an early tagging period of 1 h and a late tagging interval of 4 h, thus confirming the possibility of a switch from the CaMKII-mediated tag-setting process. The second assumption is supported by the findings that PKMζ inhibition during the priming process, or during the induction of E-LTP, prevents STC. Thus, consistent with previous study that PKMζ mediates synaptic tag setting in LTP at CA1 stratum oriens (Sajikumar et al., 2007), the present data confirmed that in certain conditions synaptic tagging can be mediated by PKMζ. It was reported recently that metaplasticity elicited by mGluR activation before the induction of a local form of LTP can generate newly synthesized PKMζ that acts as a PRP for functional plasticity (Sajikumar and Korte, 2011a). The present results extend these findings by adding the notion that RyR priming can also generate PKMζ as a plasticity factor that mediates the setting of synaptic tag in primed E-LTP. Biochemical analysis of PKMζ in the current study reveals that there is an increased amount of PKMζ following RyR activation, additionally supporting this interpretation.

4.2.2 Metaplasticity Enables a Stable Synaptic Tag Setting

It is well documented that both E-LTP expression and CaMKII mediated synaptic tag can be reset by DP 5 min after the induction of E-LTP, hence interfering with the expression of STC and long-term memory trace formation (Sajikumar and Frey, 2004a; Sajikumar et al., 2009). In contrast to that, the present data show that both RyR and mGluR primed E-LTP was resistant to DP when applied 5 min after E-LTP induction, still expressing STC. However, the tag-resetting experiments during the inhibition of PKMζ resulted in a complete DP of primed E-LTP and its STC. These results indicate that RyR or mGluR priming promotes a stable tag-setting process through PKMζ-mediated mechanisms. Of note, RyR priming does not guarantee a stable synaptic tag setting as the synaptic tag set by RyR primed STP are more fragile and less stable in comparison to the synaptic tag set by

conventional E-LTP (Sajikumar et al., 2009). It was reported previously that activity dependent local translation provides input-specific synaptic immunity against synaptic degradation which is induced by DP (Woo and Nguyen, 2003a; Sajikumar and Frey, 2004a). Thus, *de novo* protein synthesis of PKMζ by RyR or mGluR priming might prevent the degradation of the synaptic tag. In this manner, the PKMζ-mediated synaptic tag stays intact up to 4-5 h. However, a time window of 6 h was ineffective for STC, this may be due to degradation of the PKMζ-mediated tag by this late time point. It remains to be determined whether newly transcribed PKMζ can rescue synaptic tags from degradation and further prolong the STC window. Nevertheless, metaplasticity can generate "stable synaptic tags" that enable the coding of memory engrams for an extended period of time, allowing associativity in the "late" stage of LTP (Figure 4. 1).

Overall, these present data reveal that metaplasticity by RyR activation alters the synaptic tag setting from a CaMKII-mediated process to PKMζ-mediated process. Furthermore, CaMKII mediated synaptic tag setting is a fragile and "short-lived tag setting process" enabling the associative process for only a limited time period, whereas PKMζ-mediated tag setting is a stable and a "long-lived tag setting process" that extends the associativity for a longer period of time (Figure 4. 1). This proposal is consistent with the recent findings that CaMKII acts upstream to facilitate TrkB for synaptic tagging, but it is a fragile process lasting only about 60 min (Lu et al., 2011). It is also in accordance with the earlier findings that DP 5 min after the induction of E-LTP resets the synaptic tag and interferes with the expression of STC (Sajikumar and Frey, 2004a; Sajikumar et al., 2009).

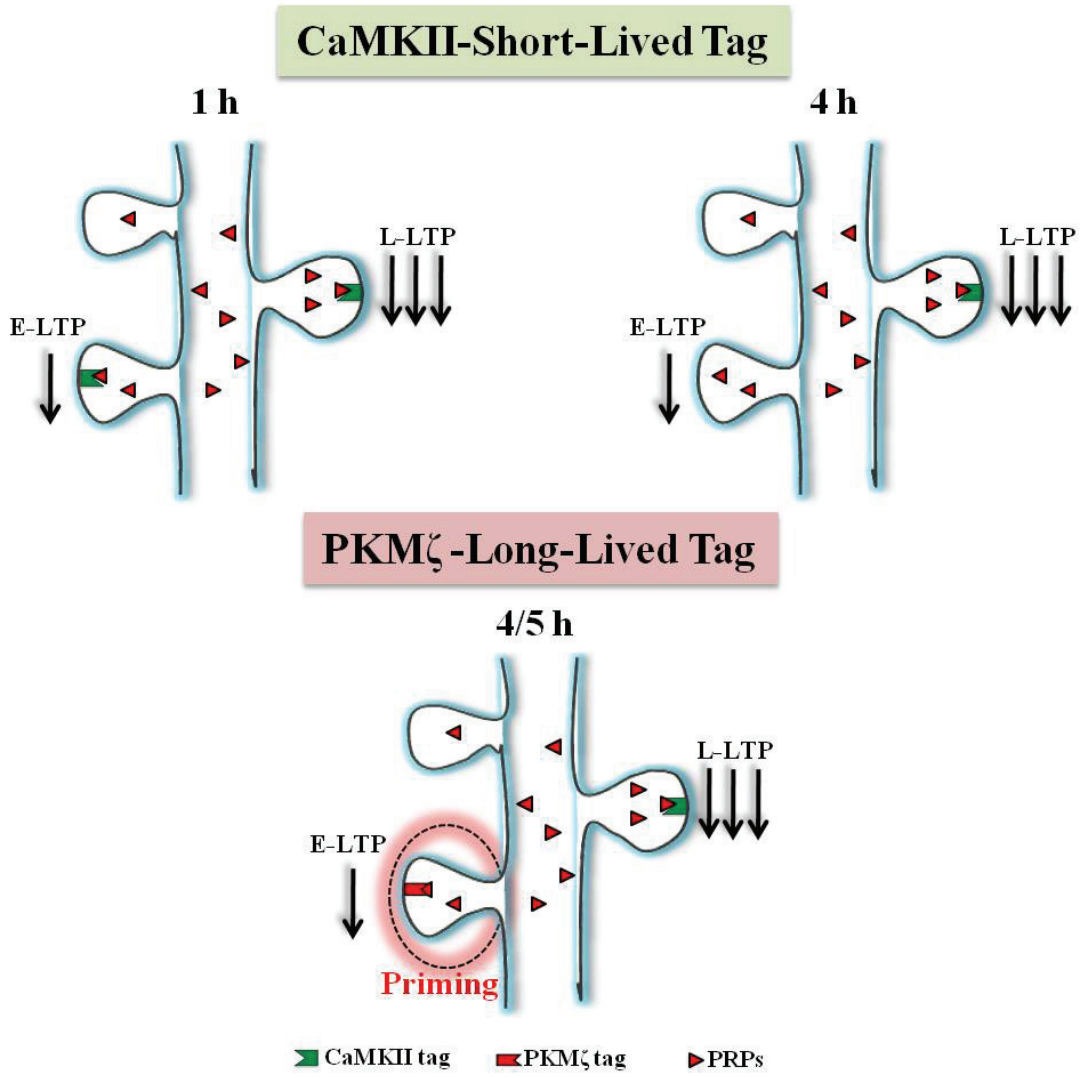


Figure 4. 1 | A scheme representing how metaplasticity promotes LTP associativity across time. **(a)** In normal, compartment-restricted STC (non-primed STC), induction of E-LTP marks synapses with a CaMKII-mediated tag (green) (Sajikumar et al., 2007; Redondo and Morris, 2011) that can last up to 60 min. A 60 min interval is an effective time period for the capture of plasticity factors such as PKMζ (red triangles) from a strongly tetanized, nearby input, that supplies plasticity factors (left panel). A 4 h interval between the induction of E-LTP and L-LTP does not promote STC, because the CaMKII-mediated tag has already disappeared (right panel). Thus the CaMKII-mediated tag is a short-lived tag, since it can mediate associative interactions only over an interval of 60 min. **(b)** In metaplasticity enabled STC (primed-STC), the molecular mechanism of the synaptic tag is altered from CaMKII to PKMζ (red), which enables those synapses to capture plasticity factors (red triangles) from a nearby synaptic input across 4-5 h. The PKMζ-mediated tag is a "long-lived tag" since it can mediate associative interactions over a longer period.

4.3 PKMζ: a Mediator of Synaptic Tags and a Plasticity-Related Protein

PKMζ is the first identified molecule that maintains the late phase of LTP (Ling et al., 2002; Serrano et al., 2005; Sacktor, 2011; but also see Lee et al., 2013; Volk et al., 2013). This is supported by the findings that PKMζ is newly synthesized by strong, but not weak tetanization (Osten et al., 1996), and postsynaptic perfusion of PKMζ potentiates the

synapse (Ling et al., 2002). Moreover, L-LTP is reversed by inhibiting the kinase, even when the inhibitors were applied many hours after the initial protein synthesis-dependent time window (Serrano et al., 2005). Strikingly, PKMζ has been identified as LTP-specific PRR which is critical for the transformation of E-LTP into L-LTP (Sajikumar et al., 2005b). The present study is consistent with these findings because continues application of PKMζ inhibitor myr-ZIP (1 μM) after the establishment of RyR primed STC disrupts not only the maintenance of L-LTP , but also the capture process of primed E-LTP. This is also in good agreement with previous findings that the tagged synapses in RyR primed STP capture PKMζ for maintenance (Sajikumar et al., 2009).

How does PKMζ enable associativity for an extended period during STC? PKMζ is believed to potentiate synaptic transmission by persistently releasing AMPARs from an extrasynaptic pool and by this means enhances NSF/GluR2-mediated trafficking (Yao et al., 2008; Migues et al., 2010). Recently, a model of "PKMζ-synaptic autotagging" has been proposed for the formation of long-term memory at the cellular level (Sacktor, 2011). According to this model, PKMζ in tagged synapses phosphorylates a substrate, possibly the GluR2 C-terminal or its associated proteins, resulting in the release of the AMPAR from protein interacting with C kinase-1 (PICK1) by NSF. This decreases AMPAR endocytosis and enables the redistribution of the extrasynaptic AMPAR to postsynaptic sites. The increased amount of GluR2 at the potentiated synapse acts as a "tag" that captures the PKMζ-PICK1 complex. The present findings are in line with this prediction, since PKMζ generated as a consequence of metaplasticity events alters the normal tag-setting process from a CaMKII-mediated one to PKMζ-mediated one which captures PKMζ as a PRP from neighboring synapses by strong tetanization (Figure 4. 2). It has been proposed that enhanced local translation of PKMζ is essential for its maintenance at the potentiated synapses, forming a positive feedback loop (Sacktor, 2012). The present observations are also in line with this hypothesis, since RyR priming triggers PKMζ synthesis that can be used for maintaining this kinase at the potentiated synapse, enhancing synaptic strength. This prediction is further supported by the biochemical evidences that PKMζ inhibition by myr-ZIP during RyR priming does not lead to a significant increase of PKMζ as that of RyR priming.

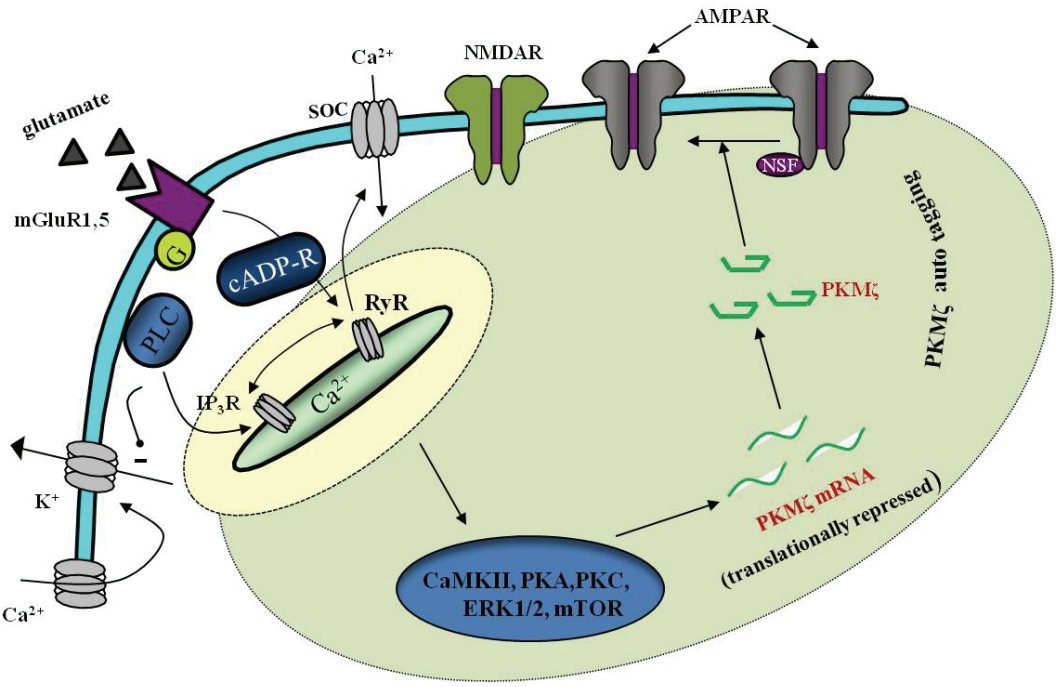


Figure 4. 2 | A model representing how PKMζ acts as a synaptic tag and a plasticity-related protein in a metaplasticity situation.

Priming of ryanodine receptors (RyRs) by ryanodine results in the release of Ca^{2+} from intracellular stores. This Ca^{2+} release is supplemented by Ca^{2+} entry into the cell through store-operated channels (SOCs) in the plasma membrane. These processes trigger the activation of kinases such as α-calcium/calmodulin-dependent protein kinase II (αCaMKII), extracellular-signal-regulated kinase 1 (ERK1), ERK2, protein kinase C (PKC) mammalian target of rapamycin (mTOR), which contributes to the activation of local protein synthesis machinery by releasing the translational block of dendritically located PKMζ (Abraham, 2008a; Sacktor, 2011). The activated PKMζ acts as a synaptic tag in the primed synapses. Newly generated PKMζ due to the strong tetanization drives GluR2 containing AMPAR subunits from the extrasynaptic part to the postsynaptic part in an N-ethylmaleimide-sensitive factor (NSF) mediated pathway (Sacktor, 2011).Thus, the PKMζ-tag captures PKMζ-PRP. IP_3, Inositol trisphosphate; IP_3R, IP_3 receptor; PLC, phospholipase C; RyR, ryanodine receptor.

STC is compartment restricted, i.e., confined to distinct dendritic branches (Alarcon et al., 2006; Sajikumar et al., 2007), and each dendritic branch further contains "synaptic units" or "clusters" (i.e., neuronal compartment on a dendritic branch) with different plasticity thresholds (Sajikumar and Korte, 2011a). Metaplasticity by group 1 mGluR activation governs the compartmentalization processes of STC by altering the plasticity thresholds of "synaptic units" or "clusters" by providing new PRPs like PKMζ and regulating PRPs like BDNF in STC. The present findings support the "synaptic unit" model for the following reasons: 1) Metaplasticity by RyR or mGluR activation alters the normal tag setting process and 2) the PKMζ-mediated tag-setting process resulted in altered plasticity thresholds for expressing STC for a longer period of time. Collectively, these findings suggest that the "PKMζ-mediated synaptic tag" can capture PKMζ-PRP generated due to the strong activity of the nearby synapses, thereby mediating associativity in the late-phase

of LTP. Thus PKMζ acts as a molecule for mediating a synaptic tag as well as a PRP for an extended period of associativity.

4.4 Metaplasticity Compensates Synaptic Memory Loss in Alzheimer's Disease

4.4.1 Synaptic Plasticity Deficits in Alzheimer's Disease

In AD, synaptic plasticity dysfunction in the hippocampus caused by abnormal accumulation of Aβ occurs at an early, pre-plaque stage and has been proposed as the best neurobiological correlate that underlies the beginning of AD pathogenesis (Hardy and Selkoe, 2002; Oddo et al., 2003; Billings et al., 2005). In the present study, hippocampal synaptic plasticity including L-LTP and L-LTD were examined in double transgenic AD mouse model of APP/PS1 mice (3-4-month old) which show fast appearance of the AD phenotype. The results show that L-LTP induced by a HFS protocol was impaired in APP/PS1 mice, resembling that of an E-LTP. This is consistent with the previous findings that exogenous Aβ application reduces LTP in hippocampus both *in vivo* and *in vitro* (Cullen et al., 1997; Lambert et al., 1998; Freir et al., 2001; Klyubin et al., 2004; Shankar et al., 2008; Li et al., 2011) and, various transgenic AD mice that overexpress Aβ show an impairment in LTP (Chapman et al., 1999; Gong et al., 2004; Gureviciene et al., 2004; Trinchese et al., 2004; Gruart et al., 2008; Ma et al., 2010). This is also compatible with the notion that Aβ instead of the amyloid plaques acts directly on the pathways involved in the formation of L-LTP (Hardy and Selkoe, 2002).

In my studies, I observed that L-LTP is impaired, and STC induced by a "strong-before-weak" experimental paradigm is absent in APP/PS1 mice. Establishment of STC depends on heterosynaptic tag-PRPs interactions (Frey and Morris, 1998b; Redondo and Morris, 2011). The impaired L-LTP in APP/PS1 mice indicates the degenerative neural network responses to the strong synaptic stimuli in a way that is incapable of generating PRPs such as PKMζ to maintain the long-lasting synaptic activity. Thus even if the synaptic tag is intact, there is nothing to be captured by the tagged synapses. This is supported by the biochemical evidences that 1) PKMζ expression level is decreased in hippocampal CA1 of APP/PS1 mice and 2) after LTP induction, PKMζ in the hippocampal CA1 of APP/PS1 mice does not increase as much as that of WT mice. As PKMζ is essential for the consolidation of LTP and STC, the decreased amount of PKMζ may

explain both LTP and STC deficits in APP/PS1 mice.

L-LTD in APP/PS1 mice induced by strong LFS show the same time course as that of WT mice. This is in agreement with the earlier findings that application of Aβ has no effect on hippocampal LTD that induced by a stronger protocol (Wang et al., 2002; Raymond et al., 2003; Li et al., 2009). Despite L-LTD is long-lasting, no cross-capture is observed in APP/PS1 mice. This can be interpreted as follows: 1) L-LTD in the hippocampus of APP/PS1 is not a conventional LTD that can provide PRPs such as PKMζ for the tagged synapses that have received E-LTP induction and 2) The synaptic tag of LTP is impaired in APP/PS1 mice, which limits the ability of the activated synapses to capture the PRPs from the nearby heterosynaptic input of L-LTD. It was reported recently that NMDAR-dependent LTD in the Aβ-treated hippocampal slices is not a conventional LTD that follows a canonical p38 MAPK pathways (Li et al., 2009). MAPK cascade is a biochemical signal integration system that regulates neuronal activity-induced translation and subserves synaptic plasticity and memory (Sweatt, 2001; Thomas and Huganir, 2004a). Strikingly, MAPK cascade is essential for mammalian associative learning (Atkins et al., 1998). Thus, the absence of cross-capture in APP/PS1 mice may be attributed to the lack of PRPs such as PKMζ that normally produced by L-LTD. This prediction is further supported by the finding that synaptic tag setting process is preserved in APP/PS1 mice as the expression of E-LTP is normal (Figure 2. 1).

4.4.2 Metaplastic Upregulation of PKMζ Compensates Synaptic Plasticity Deficits in Alzheimer's Disease

The present study show that metaplasticity by pharmacological activation of RyR activation via its agonist RYA (10 μM) prevents the synaptic plasticity deficits in APP/PS1 mice, including L-LTP, STC and cross-capture. Strikingly, the maintenance of the rescued L-LTP, STC and cross-capture in APP/PS1 mice requires persistent PKMζ phosphorylation, as the same as that of the conventional ones, indicating that metaplasticity restores the impaired synaptic plasticity by utilizing newly synthesized PKMζ as PRP.

The present data reveal that RyR priming reverses synaptic plasticity deficits in APP/PS1 mice through metaplastic upregulation of new protein synthesis. This is in accord with the findings that metaplasticity by RyR or mGluR activation facilitate LTP by new synthesis of PRPs that leads to tagging and capture (Sajikumar and Korte, 2011a; Li et al., 2014). It is also in line with the findings that synaptic plasticity and memory dysfunctions in AD could be restored by pharmacological interventions that target the protein synthesis pathway

(Vitolo et al., 2002; Tully et al., 2003; Gong et al., 2004; Comery et al., 2005; Puzzo et al., 2009). For instance, brief treatment with the phosphodiesterase 4 (PDE4) inhibitor rolipram that increases cAMP levels and further stimulates the cAMP/PKA/CREB pathway can ameliorate both LTP and contextual learning deficits in the double-transgenic APP/PS1 mice (Gong et al., 2004). Rolipram exerts its effects in the neural network of AD by reversing the decrease in CREB phosphorylation, indicating synthesis of plasticity proteins mechanisms is involved (Tully et al., 2003; Gong et al., 2004). Remarkably, rolipram-reinforced E-LTP in the hippocampal CA1 region is protein synthesis-dependent and resembles a conventional L-LTP which can provide PRPs thus allowing synaptic capture by the tagged synapses (Navakkode et al., 2004). Rolipram is currently being tested as a therapeutic agent for preventing memory loss in the early-stage of AD patients (Tully et al., 2003). In addition, a recent study showed environmental novelty exploration that is involved in the induction of the synthesis of PRPs potently protects against Aβ oligomer-mediated synaptic dysfunction (Li et al., 2013). Similarly, up-regulation of mTOR signaling that is critical for controlling mRNA translation by both pharmacological and genetic methods prevents Aβ-induced synaptic impairments (Ma et al., 2010). It was shown that protein synthesis machinery restores synaptic dysfunction in AD without altering pathology (Comery et al., 2005). Nevertheless, it stabilizes or optimizes the synaptic circuitry in the early phase of AD (here 3-4-month old APP/PS1 mice), thus delaying or preventing the progression of the disease.

The current data identified PKMζ as one of the newly synthesized proteins through RyR priming and, plays an essential role in the rescue of L-LTP and STC in the neural network of AD. Biochemical evidences show that PKMζ expression is decreased in APP/PS1 mice, at least in hippocampal CA1. The decrease of PKMζ in APP/PS1 mice may be related to some biochemical signaling alterations such as CaMKII, PKA, MAPK, phosphatidylinositol 3-kinase (PI3K), mTOR, as well as actin filament formation, all of which act in concert to increase PKMζ synthesis in normal conditions (Sacktor, 2008, 2011). Indeed, it was reported that both PKA activity and CREB phosphorylation in response to glutamate is decreased in cultured hippocampal neurons treated with sublethal levels of Aβ42 (Vitolo et al., 2002). Consistent with this, a decrease of CREB phosphorylation was observed in the hippocampal CA1 of APP/PS1 mice (3-month old) (Gong et al., 2004). In addition, inhibition of mTOR signaling was reported in hippocampal slices of AD models and in WT hippocampal slices exposed to exogenous Aβ1-42 (Lafay-Chebassier et al., 2005; Ma et al., 2010). Not only the expression, but the

function and distribution of PKMζ in AD are altered. PKMζ perpetuates both LTP maintenance and long-term memory trace mainly because it can block NSF/GluR2-dependent pathway that removes postsynaptic GluA2-containing AMPARs, resulting in a persistent increase of these receptors at postsynaptic sites (Yao et al., 2008; Sacktor, 2011). However, in the AD brain tissue it is observed that PKMζ aggregates with NFTs restricted to limbic or medial temporal lobe structures such as HF, EC, and amygdala, which may inhibit the normal activity of this kinase in modulating the trafficking of AMPARs at synapses (Crary et al., 2006). In addition, subcellular distribution of GluA2 and PKMζ is altered in the aging brain in that there is a decreased density of synaptic GluR2 in large dendritic spines coexpressing PKMζ, and this decrease correlates with impaired recognition memory (Hara et al., 2012). The present data show that RyR priming reverses synaptic dysfunction in APP/PS1 mice through metaplastic upregulation of PKMζ, which is in line with previous report that PKMζ overexpression in the neocortex could enhance an associative type of LTM – conditioned taste aversion (Shema et al., 2011). But after its synthesis by RyR activation, how can PKMζ activity persistent despite of the turnover of individual PKMζ molecules? It is proposed that PKMζ synthesis are maintained by positive feedback loops, thereby perpetuating the consolidation of LTP and LTM (Sacktor, 2011). In particular, PKMζ phosphorylation of protein interacting with NIMA1 (PIN1; suppress PKMζ mRNA translation in basal conditions) upregulates the translation of PKMζ – a positive loop (Westmark et al., 2010). The current findings are in compatible with this proposal, as biochemical evidence show that PKMζ inhibition by myr-ZIP during RYA priming in APP/PS1 mice does not lead to a significant increase of PKMζ as that of the pure RYA priming, whereas the control peptide of myr-ZIP, scr-ZIP does. In short, these results reveal that RyR priming reverses the impaired synaptic plasticity in APP/PS mice by metaplastic upregulation of PKMζ. Thus, it may be speculated that metaplastic upregulation of PKMζ could prevent synaptic degradation in the neural network of AD. These finding also supports the hypothesis that PKMζ can be employed as an attractive potential therapeutic target for preventing or treating age-related memory decline (Aicardi, 2013).

RyR activation by its agonist ryanodine or caffeine has been suggested to ameliorate the dysregulated synaptic deficits in AD. Key evidence for this hypothesis is that acute RYA (0.2 μM) application in the hippocampal slices rescues stressed experience-induced LTP deficit in triple-transgenic mice of AD (Grigoryan et al., 2014). And chronic caffeine feeding to APP/PS1 mice reverses memory impairment (Han et al., 2013). Interestingly,

epidemiological studies in humans have revealed that significant caffeine intake (≥ 3 cups per day) during middle-age protects against cognitive impairment and AD in old age (Ritchie et al., 2007; van Gelder et al., 2007; Eskelinen et al., 2009). In accord with those, high blood caffeine levels are directly linked to lack of progression to dementia in mild cognitive impairment (MCI) patients (Cao et al., 2012). Overall this is in line with the current findings that priming the hippocampal synapses with RyR agonist ryanodine (RYA; 10 μM) rescues synaptic deficits in APP/PS1 mice. Of note, it was reported previously that RyRs are increased in expression and function in the hippocampal neurons of AD mouse models (including APP/PS1 mice) and in post-mortem hippocampal specimens from early-stage AD patients, which are once thought to contribute to dysregulated endoplasmic reticulum (ER) calcium Ca^{2+} homeostasis that underlies synaptic loss and impaired cognitive function in AD (Bezprozvanny, 2009; Supnet and Bezprozvanny, 2010; Oules et al., 2012). However, a recent elegant study by Liu et al reported that a dual role for elevated level of RyR3 in AD pathology: in young AD neurons (≤ 3-month APP/PS1 mice), the enhanced expression of RyR3 protects AD neurons from synaptic and network dysfunction, whereas in older AD neurons (≥ 6-month APP/PS1 mice), increased RyR3 activity contributes to pathology (Liu et al., 2014). Thus, pharmacological activators of RyR may be beneficial when used prior to AD disease onset or in its initial stages by stabilizing neuronal activity. My findings support these observation and in addition the proposal that Ca^{2+} stores may be a therapeutic target for AD in the early stage (Chakroborty and Stutzmann, 2014).

4.5 Roles of Metaplasticity in Neuronal Network Function

Synaptic plasticity such as LTP and LTD is demonstrated in many aspects as a cellular mechanism for information storage in the brain provided that it is properly regulated. Unregulated Hebbian plasticity (LTP) could lead to extreme levels of potentiation that further may cause excitotoxicity, while too much LTD can render the neural network incapacitated or even nonfunctional. Metaplasticity mechanisms could homeostatically adjust the threshold for both LTP and LTD induction in correspondence with previous postsynaptic activity, thereby preventing the saturation of LTP and LTD (Abraham and Tate, 1997; Abraham, 2008). In line with this, in the present study I could show that the threshold of LTP induction is decreased with metaplastic RyR or mGluR activation. In this manner, synaptic weights can be maintained within a dynamic range so that LTP and LTD

are kept online and thus flexible to the learning process (Hulme et al., 2013).

Metaplasticity integrates synaptic events together across time scales (from minutes to hours, even days to weeks) (Abraham, 1999) , which is longer than a typical associative synaptic plasticity that only lasts 60 min *in vitro*, collectively influencing the ultimate duration and direction of the expressed synaptic plasticity. Notably, the duration of associativity itself can be regulated by metaplasticity mediated mechanism such as prior RyR activation (Sajikumar et al., 2009). The present data provide compelling proof that metaplasticity could prolong the duration of associativity up to 5 h. Thus, metaplasticity serves to extend the time course of associativity between neural events and may therefore provide neural basis for the increased information coding of spaced learning trails or trains of conditioning stimuli. In addition, theoretical models of dynamically learning neural networks predicts that incorporating multiple metaplastic states into the functionality of the synapses prolongs the duration of memories stored in the network (Fusi et al., 2005). Indeed, inducing metaplasticity *in vivo* such as novelty exposure has been proved to reinforce hippocampus-dependent memory consolidation (Duffy et al., 2001; Wang et al., 2010b; Almaguer-Melian et al., 2012).

Metaplasticity also contributes to protect against pathological changes of neuronal network, in which strong stimulation of a fragile synapse is not valid to realize any gains of function. One rather convincing example is that following chronic monocular visual deprivation (MD) that usually leads to loss of visual acuity, manipulating the NMDAR subunits composition (lowering the NR2A:NR2B ratio) through dark rearing (3-10 days) could reinforce weak cortical inputs in visual cortex (He et al., 2007). Additionally, metaplastic upregulation of PRPs by novelty exposure has been reported to restore hippocampal plasticity deficits in neurodegenerative diseases such as AD (Li et al., 2013). In line with these studies, the current study show metaplastic upregulation of PRP like PKMζ could ameliorate the impaired synaptic plasticity in AD. Hence, metaplasticity may compensate the loss of function in the abnormal neural network and prepare a permissive milieu that arguments the subsequent neural plasticity.

4.6 The Specificity of ZIP on PKMζ

Sustained activity of the brain-specific PKMζ has been reported to be essential for maintaining L-LTP and LTM in both the hippocampus and cortex (Sacktor, 2011). This is revealed by the findings that inhibiting the PKMζ activity by its inhibitor ZIP reverses previously established LTP and memory storage both *in vivo* and *in vitro* (Ling et al., 2002; Sajikumar et al., 2005b; Serrano et al., 2005; Pastalkova et al., 2006; Serrano et al., 2008). In good accord with these findings, the current study shows that the application of ZIP at a concentration of 1 μM prevented the persistence of L-LTP and STC in the hippocampal slices *in vitro*. However, several recent studies questioned the specificity of ZIP. One study showed that ZIP at 1 μM fails to inhibit PKMζ in brain slices overexpressing PKMζ (Wu-Zhang et al., 2012). Nevertheless, subsequent work disputed that this may be partially due to the overexpression system they used in all of their experiments (Yao et al., 2013). Moreover, two recent genetic studies raised the doubt further showing that ZIP reverses established LTP and memory not only in WT mice but also in constitutive PKC/PKMζ KO mice (Lee et al., 2013; Volk et al., 2013). Notably, these constitutive PKC/PKMζ KO mice show intact LTP and perform normal in a hippocampus-dependent learning and memory tasks (Lee et al., 2013; Volk et al., 2013). The two reports provide evidence that ZIP functions in erasing LTP and memory by PKMζ-independent mechanisms and probably inhibits an alternative target that sustains LTP and LTM when PKMζ is absent. Importantly, they bring into question the role of PKMζ in the maintenance of LTP and memory.

Despite these findings of Volk (2013) and Lee (2013), we cannot conclusively rule out the possibility that PKMζ is a key player that maintains LTP and LTM. This is because on the one hand, genetic manipulations that lead to PKMζ overexpression after learning enhances memory (Drier et al., 2002; Shema et al., 2011), whereas an inhibitory form of PKMζ following training is sufficient to erase the established memory (Shema et al., 2011). One the other hand, redundancy of signaling pathways are involved in synaptic plasticity or memory – hundreds of molecules are involved following LTP/LTD induction, whereby appropriate molecules could compensate each other's deficiency (Sanes and Lichtman, 1999). A similar redundancy between aPKC isoforms is found (Price and Ghosh, 2013). Among the aPKC isoforms, PKCλ is widely expressed in the nerve system such as hippocampus and cortex, and importantly, its amino acid levels share 86% identity with PKMζ (Standaert et al., 2001; Bosch et al., 2004; Jiang et al., 2006; Ren et al., 2013). Hence, it can be speculated that PKCλ/ι accounts for the signaling redundancy. Indeed,

preliminary data from Sacktor laboratory show when PKMζ is knocked out constitutively, there are compensatory increases in PKCλ phosphorylation (Tsokas. et al., Society for Neuroscience Annual Meeting, New Orleans, LA, 2012), and additional unpublished data using a "split-brain" preparation showed that a ~400% increase in PKCλ phosphorylation in the contralateral isolated hippocampus (Tsokas. et al., Proceedings of the 9th FENS Forum of Neuroscience, Milan, Italy, 2014). Strikingly, activation of PKCλ is essential for LTP expression in the hippocampal slices (Ren et al., 2013), which may account for the normal expression of LTP in constitutive PKC/PKMζ KO mice. However, more studies will be needed to investigate whether PKCλ has a role in maintaining memory.

The molecule that ZIP targets except PKMζ is not known. One possibility is that ZIP inhibits PKCλ which contains the same pseudosubstrate sequence (myr-SIYRRGARRWRKL) as PKMζ/PKCζ (Jiang et al., 2006) (also see Figure 2. 8). This is supported by the evidence that ZIP at concentration of 2.0 μM competitively inhibit the activity of both PKMζ and PKCλ (Ren et al., 2013). In the study of Lee et al., (2013), the authors use ZIP at a concentration of 4-5 μM which may very likely targets PKCλ due to the compensatory increase in the PKMζ-deficient mice. Studies that used a high concentration of ZIP should be interpreted cautiously. Future study will be needed to examine the extent to which ZIP inhibits PKMζ and/or PKCλ.

Though the specificity of ZIP for inhibiting PKMζ is controversial in terms of plasticity in some brain regions but its role in maintaining memory in insular cortex (IC) is not questioned (Shema et al., 2007; Shema et al., 2009; Shema et al., 2011). Additionally, there are preliminary evidence showing that constitutive PKMζ KO mice display altered learning pattern and conditional PKMζ KO mice have deficits in spatial LTM (Tsokas. et al., Proceedings of the 9th FENS Forum of Neuroscience, Milan, Italy, 2014).), indicating again that PKMζ is in one way or another crucial for the maintenance of L-LTP and spatial LTM under physiological conditions or when compensation is avoided.

4.7 Conclusion and Outlook

In the present study, a novel form of metaplasticity – RyR activation – was identified in the hippocampus. Metaplasticity mediated by RyR activation is physiological relevant, showing the same effect as that of the synaptic mGluR activation form induced by synaptically released glutamate. By metaplastic upregulation of the "memory molecule" – PKMζ, RyR or mGluR priming has substantial effects on the functional plasticity in the hippocampal neural network.

The data presented in the initial part of the thesis provide a candidate mechanism for how activated neural network (here RyR or mGluR activation) can initiate and maintain memory for an extended period of time. Based on the experimental model of synaptic tagging and capture (STC) that are widely accepted to be the cellular basis of associative long-term memory (LTM) formation, the current study firstly show that the association of weak synapses with strong synapses in the "late" stage (i.e., time interval of 4 h) fails to occur, confirming previously published results. This is due to the decay time course of the synaptic tag, which lasts only 1 h *in vitro*. However, priming the hippocampal neurons either by RyR activation via its agonist ryanodine (RYA; 10 μM) or synaptic group 1 mGluR activation via 2xTBS prolongs the durability of the synaptic tag to 5 h, thus extending the time window for associative interactions mediating storage of LTM. Furthermore, RyR priming alters the synaptic tag setting from CaMKII-mediated process to PKMζ-mediated process. Intriguingly, the PKMζ-mediated synaptic tag in RyR primed STC is immune to depotentiation (DP), indicating that it is a stable synaptic tag resistant to disruption. Thus the association of weak synapses with strong synapses in the "late" stage of associative memory formation occurs only through metaplasticity. The current findings indicate that the initial fingerprint of memory, the synaptic tag, is a dynamic molecular complex that can alter its mechanisms based on previous neuronal activity. Metaplasticity can tune activated neuronal networks for coding stable memory engrams for an extended period of time by switching the synaptic tag from a "fragile" state to a more "stable" state. It would be interesting to further investigate whether such metaplasticity form can be used to expand the temporal interval of associations during behavior.

The second part of the work revealed a critical role of metaplasticity in preventing the degradation of synaptic memory in Alzheimer's disease (AD). Synaptic plasticity dysfunction in the hippocampus best correlates with learning and memory deficits in AD. The present data show that hippocampal synaptic plasticity such as L-LTP, late

associativity process of STC and cross-capture was absent in an AD mouse model of APP/PS1 mice (3-4-month old). Intriguingly, priming the hippocampal neurons by RyR activation via its agonist ryanodine (10 μM) reverses these synaptic plasticity deficits and restores them to normal levels (at least in the requirement for PKMζ activity for maintenance), thus preventing the decay of memory. Moreover, biochemical evidences in the current study show that there is decreased amount of PKMζ expression in the hippocampal CA1 of this mouse model, which may account for the impaired synaptic plasticity. Strikingly, prior activation of the hippocampal neural network by RyR activation upregulates PKMζ expression, thereby rescuing the impaired synaptic plasticity. Thus, metaplasticity can tune the activated neural network for preventing the degradation of memory through compensatory increase of plasticity factors such as PKMζ. In the future, it would be interesting to study whether such metaplasticity can ameliorate the learning and memory deficits in live AD models.

Overall, the present data provide compelling proof that neural circuits have the robust ability to associate synaptic events (here RyR or mGluR activation) at one point in time with a later plasticity-inducing event (here LTP induction), which greatly expands a network's capacity for associating stimuli across time. Memory encoding and consolidation are not exclusively determined by the characteristics of neural stimuli, but rather it can be influenced dramatically by the neural events happening before memory encoding (i.e., metaplastic events). Through metaplasticity, the capacity of a neural network for memory encoding can be enhanced further than previously understood.

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